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Doctor of Philosophy Degree  
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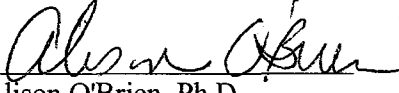
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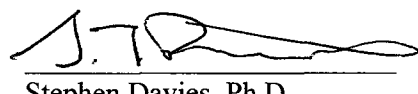
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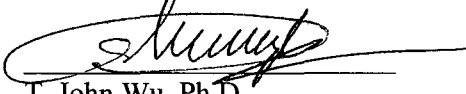
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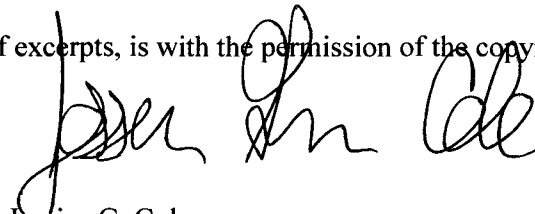
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# Abstract

Title of dissertation: “Evaluation of *Neisseria gonorrhoeae* opacity (Opa) protein loops as targets for passive vaccination and investigation of the role of Opa proteins during infection of a female host”

Jessica G. Cole, Doctor of Philosophy, 2009

Thesis Directed by: Ann E. Jerse, Ph.D. Professor

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*Neisseria gonorrhoeae* opacity (Opa) proteins mediate adherence and invasion of host cells, resistance to complement, and immunosuppression. Presented here are studies on the potential of conserved Opa protein loops as targets for passive vaccination and investigations into the role of Opa proteins during infection of the female genital tract. We demonstrated antibodies that target conserved Opa loops, the semi-variable (SV) and fourth loop (4L), recognized antigenically distinct Opa proteins. Antibodies to cyclic peptides that correspond to the SV loop (Ab<sub>SV cyclic</sub>) recognized more Opa variants than antibodies generated against linear SV-loop peptides (Ab<sub>SV linear</sub>). Ab<sub>SV linear</sub> and Ab<sub>SV cyclic</sub>, but not Ab<sub>4L linear</sub>, bound intact gonococci. Ab<sub>SV cyclic</sub>, but not Ab<sub>SV linear</sub> agglutinated bacteria and antibodies to a hypervariable loop 2 cyclic peptide (Ab<sub>HV2BD cyclic</sub>) agglutinated and blocked interactions with cervical cells. Ab<sub>HV2BD linear</sub> and Ab<sub>HV2I linear</sub> were bactericidal, and this bactericidal activity was increased 8-fold when a non-

human source of complement was used. Preincubation of Opa loop-specific antibodies with homologous variants did not protect mice from colonization; however protection studies were technically limited as 50% of the antibody was shed within one hour post-administration.

Our second objective investigated the role of Opa proteins during infection of a female host. Previous work demonstrated cyclical recovery of Opa variants and selection for Opa-positive variants in the early phase after inoculation of estradiol-treated mice with predominantly Opa-negative gonococci. Here, we demonstrated cyclical recovery in estradiol-treated and untreated mice but not in ovariectomized mice. The early phase advantage for Opa-positive gonococci was confirmed by the increased recovery of Opa-expressing gonococci compared to Opa-deficient gonococci on day 1. Additionally, Opa proteins confer an advantage for persistent infection in that 69% and 31% of mice were colonized by the Opa-expressing strain and the Opa-deficient strain on day 14, respectively. The observed fitness advantage for the Opa-positive strain is unlikely due to increased association with murine epithelial cells or resistance to complement, despite evidence that Opa-positive gonococci are more resistant to normal human sera and mucosal C3 levels fluctuate in estradiol-treated mice. We conclude another hormonally regulated factor likely provides the advantage for Opa-positive gonococci.

# Title Page

“Evaluation of *Neisseria gonorrhoeae* opacity (Opa) protein loops as targets  
for passive vaccination and investigation of the role of Opa proteins during  
infection of a female host”

By

Jessica Giddings Cole

Dissertation submitted to the Faculty in the Department of Microbiology and  
Immunology in the Emerging Infectious Diseases Graduate Program of the Uniformed  
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## **Dedication**

I would like to dedicate this work to my grandfather, Thomas A. Luther, who has been a very important part of my life and my education. He did not have the educational opportunities that he wanted and, as such, he made it a priority to ensure that his children and his grandchildren knew the value of education. He encouraged us from a very young age and did his best to help us along our chosen paths. His influence in our family has been profound and is exemplified by the fact that all of his adult grandchildren now possess terminal degrees in their field.



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# Chapter 1: Introduction

## *Background and public health implications of Neisseria gonorrhoeae infections*

*Neisseria gonorrhoeae* is a Gram-negative pathogen that was first described by Albert Neisser in 1879 as the causative agent of gonorrhea. *N. gonorrhoeae* is a human-specific pathogen that is transmitted mainly through sexual contact with an infected partner and is a disease primarily of mucosal surfaces. This bacterium is characteristically observed in pairs, or diplococci, and is catalase-positive. While the bacterium was not identified until 1879, the disease gonorrhea is described in written texts dating back to 2500 B.C. (113).

The disease gonorrhea can vary from asymptomatic to severe, however most infections with *N. gonorrhoeae* are uncomplicated lower urogenital tract infections that are often accompanied by a purulent exudate at the site of infection. This exudate is comprised of polymorphonuclear lymphocytes (PMNs), many of which contain intracellular diplococci (87). The other pathogenic species in the Neisseriaceae is *Neisseria meningitidis*, which colonizes the nasopharynx and can invade across the endothelium to cause septicemia and meningitis. The gonococcus can invade into the bloodstream and does so in 1-3% of patients to cause a disseminated gonococcal infection (DGI). DGI most often presents as dermatitis or septic arthritis but can rarely cause endocarditis or meningitis (149). While antibiotic treatment is available for *N. gonorrhoeae* there is no vaccine for this sexually transmitted pathogen, and many strains are resistant to antibiotic treatment.



Gonorrhea is a burden to the public health system and directly impacts reproductive health, especially in women. Globally it is estimated that there are over 62 million cases of gonorrhea annually (61) and more than 350,000 cases were reported in the United States (US) in 2006 (125). While most infections are uncomplicated, ascension can occur in both men and women. Ascended infections in men most commonly cause epididymitis which, if left untreated, can cause sterility. Lower urogenital infections in men are more likely to be symptomatic than women, and it is estimated that 30-80% of cervical infections are asymptomatic or subclinical. As such, many women do not seek early treatment, and there are many complications associated with chronic infections (11, 122, 137). It is estimated that 20% of cervical infections cause complications such as pelvic inflammatory disease (PID), salpingitis, or ectopic pregnancy. Ectopic pregnancy is a significant cause of mortality, especially in the developing world (87). In the US, infertility is a common complication of PID with approximately 1 in 6 women infertile due to the effects of PID on the reproductive system (88). In addition to direct morbidity and mortality in infected individuals, gonorrhea is a significant public health burden. Estimates suggest that over 1.8 billion dollars were spent to treat PID and its complications in the US in 1998 (148) and the cost of treating gonorrhea in patients aged 15-24 in the US was estimated to be 77 million dollars in 2000 (31). Thus, gonococcal infections are a tremendous burden on the healthcare system and development of a vaccine is of great public health importance.

In addition to the direct complications discussed above, *N. gonorrhoeae* and several other sexually transmitted pathogens are associated with increased transmission of the human immunodeficiency virus (HIV) (36, 48, 108, 191). Individuals are 2-5

times more likely to acquire HIV if they already have another sexually transmitted infection (STI) (56). This increased risk of HIV acquisition is not simply due to increased high-risk behavior, but there is a biological predisposition to HIV infection when other STIs are present in the genital tract. Reduction of the prevalence of *N. gonorrhoeae* could, therefore, potentially be a public health strategy to decrease the number of HIV-infected individuals, and in fact, control of STIs was shown to decrease the HIV prevalence in Tanzania by 40% (69). Therefore, development of a gonococcal vaccine could also help slow the global spread of HIV.

### *Overview of gonococcal pathogenesis*

*N. gonorrhoeae* is a human-specific pathogen whose co-evolution with humans has resulted in an exquisitely well-adapted mucosal pathogen. Unlike bacterial pathogens that secrete toxins or directly invade and destroy host cells, the pathogenicity of the gonococcus is subtle and many infections, especially in women, are asymptomatic or subclinical (87). The balance between establishing a persistent infection while avoiding the immune response is essential to the long-term success of this pathogen. As such, the ability to vary virulence factors not only allows for adaptation to multiple body sites but potentially allows evasion of the host immune response. It is estimated that over 82 genes predicted by the genome sequence of strain FA1090 are phase variable, including genes involved in adherence and invasion (*pilE*, *pilC1*, *pilC2*, *opa*), iron acquisition (*hpuA*), serum resistance (*lgtA*, *lgtC*, *lgtD*, *lgtG*, *opa*), and those that encode hypothetical proteins (170). In comparison, the phase variable gene repertoires of *Helicobacter pylori*

and *Haemophilus influenzae* are predicted to consist of 46 and 12 genes, respectively (86, 154).

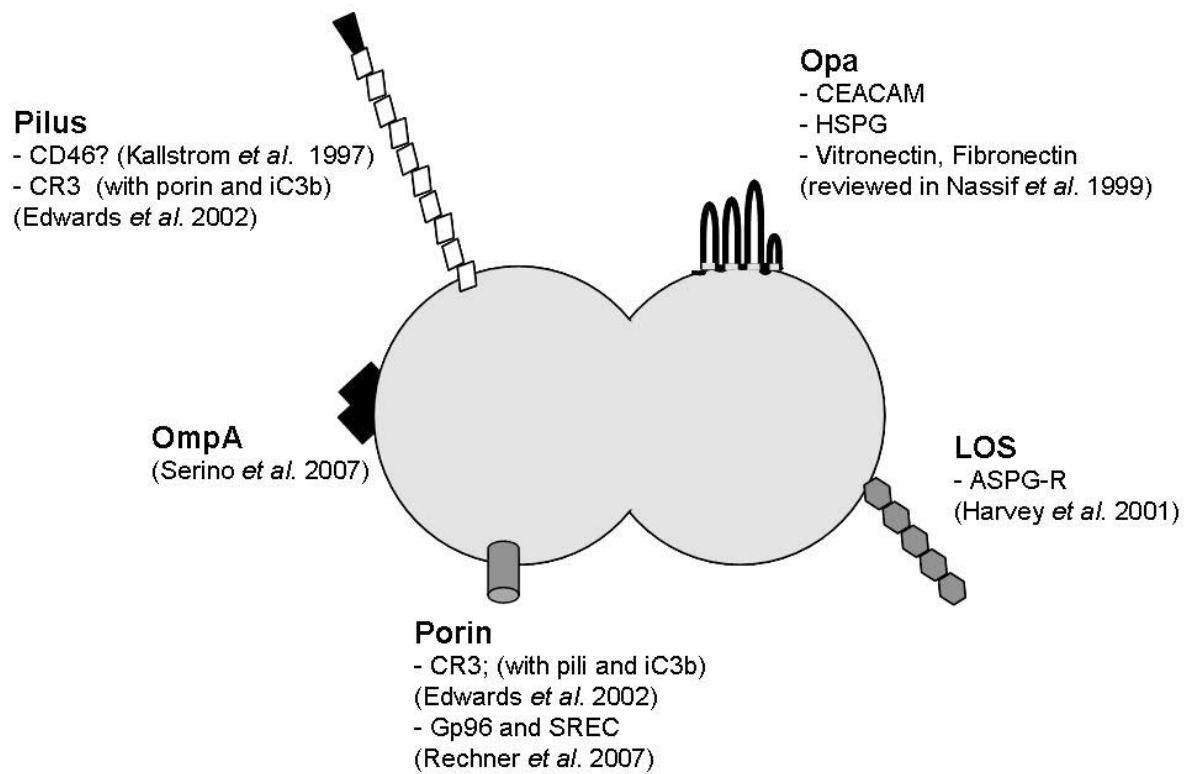
### ***Colonization factors***

The first step to establish colonization for any mucosal pathogen is adherence to host cells. Several outer membrane structures mediate adherence of *Neisseria* to human cells including colonization pili, opacity (Opa) proteins, and other outer membrane structures. These factors and their receptors are depicted in Figure 1.

The first well-described adherence protein identified in the gonococcus was the type IV pilus, which promotes bacterial adherence to host cells and is also involved in twitching motility, transformation and bacterial aggregation (126). Neisserial pilin, which is the major subunit of the pilus, is antigenically variable due to recombination events that occur between the *pilE* gene and the *pilS* storage loci (82). The expression of pili is also phase variable due to several different mechanisms. Inaccurate recombination events between *pilE* and *pilS* can produce a non-functional protein, and deletions or duplications within *pilE* prevent pilin polymerization and assembly (115). A final mechanism of pilus phase variation occurs when frameshift mutations occur in a poly-G tract upstream of *pilC*, which encodes a protein that is required for pilus assembly (98). PilC, which has been localized to the bacterial membrane and tip of the pilus structure (143, 153), was recently shown to interact with CD46 on host cells (99). The role of this interaction is still controversial as other researchers have not confirmed the role of CD46 in mediating pilus-dependent adherence (103, 126). After initial adherence by the

**Figure 1. Adherence factors expressed by *Neisseria gonorrhoeae*.** Shown are the known adherence factors expressed by the gonococcus with host receptors listed beneath each adherence ligand, if known. The expression of redundant virulence factors allows the gonococcus to adapt to different anatomical sites. Antigenic or phase variation of pili and Opa proteins allows evasion of the host immune response while still retaining the ability to colonize the mucosa. Abbreviations: CR3; complement receptor 3; CEACAM, carcinoembryonic antigen-related cellular adhesion molecule; HSPG, heparan sulfate proteoglycan; ASGP-R, asialoglycoprotein receptor; Gp96, glycoprotein 96; SREC, scavenger receptor expressed in endothelial cells.

Figure 1. Adherence factors expressed by *Neisseria gonorrhoeae*.



type IV pili, the opacity (Opa) proteins are thought to promote a more intimate interaction with host cells, followed by invasion into the cell. The role of Opa proteins as colonization factors has been extensively studied *in vitro* and will be discussed in detail below.

While pili and Opa proteins are thought to be important in gonococcal adherence and invasion of cells, several other adherence and/or invasion pathways are employed by the gonococcus. For example, recently, Serino *et al.* showed that an OmpA-like protein is expressed by *N. gonorrhoeae* and promotes invasion of human cells. In collaboration with Serino *et al.*, our lab showed that OmpA also provides a colonization advantage in the lower genital tract of female mice (162). Invasion into male urethral epithelial cells, but not cervical epithelial cells, occurs through interactions of certain lipooligosaccharide (LOS) species with the human asialoglycoprotein receptor (ASGP-R) (51, 75). In addition to the traditional interaction between a single bacterial ligand and a host cell receptor, *N. gonorrhoeae* can adhere to and invade host cells via a coordinate interaction between porin, pilus and iC3b deposited on LOS. Porin, pilus and iC3b are then capable of binding to human complement receptor 3 (CR3), which is present on primary cervical epithelial cells, but not primary male urethral cells (53) (52). Interaction with CR3 does not induce an inflammatory response in the host, which may explain why women are more likely to be asymptomatic than men. Gonococcal porin has two allelic forms *porB1a* or *porB1b*, which encode PIA or PIB protein, respectively. PIA strains are associated with DGI and recent work has shown that interaction of PIA, but not PIB, with human heat shock glycoprotein Gp96 promotes gonococcal adherence. Subsequent interaction of Gp96 with the scavenger receptor SREC allows invasion of cells in a low

phosphate-dependent manner (106, 147, 181). The redundancy of adherence factors expressed by *N. gonorrhoeae* presumably allows for colonization at varied sites both within the genital tract (i.e. the urethra and cervix) and outside the genital tract (i.e. the rectum, conjunctiva and pharynx). This redundancy ensures that there is a population of bacteria able to adhere after transmission from one body site to another or promote invasion through the mucosa.

### ***Iron acquisition***

Iron acquisition during infection is an essential adaptation mechanism of most parasites and has been extensively studied as a virulence factor for *N. gonorrhoeae*. The gonococcus does not encode any known siderophores, and instead, iron is acquired by binding iron coupled to host carrier molecules or by direct binding of xenosiderophores produced by commensal bacteria (159, 192). The ability to use iron from transferrin and lactoferrin is host-restricted, in that only human lactoferrin and transferrin support gonococcal growth. Consistent with the many redundant colonization factors encoded by the gonococcus, the acquisition of host-sequestered iron is facilitated by several different outer membrane receptors. All gonococcal strains have a transferrin receptor and a phase variable hemoglobin receptor, while some strains also express a lactoferrin receptor (reviewed in (159)). The role of Fe-binding proteins during infection has been studied in the male volunteer model, and the transferrin receptor was required for urethral infection with strain FA1090, which naturally lacks a lactoferrin receptor (39). In competitive infections with strains that express only the transferrin receptor or both the lactoferrin and transferrin receptors, the strain that expressed both receptors had an advantage in colonization of the male urethra (6). These results suggest that the redundancy of iron-

binding proteins provides an advantage to gonococci. The hemoglobin receptor is not required for infection; however, its expression is upregulated in women during menstruation and may provide a colonization advantage at this time (7). Our laboratory has shown that in the murine female genital tract, the transferrin, lactoferrin or hemoglobin receptors are not essential for colonization of female mice. These results suggest that other sources of iron, such as iron bound to xenosiderophores or iron complexed to various metabolites, may be available in the female genital tract (93, 128). In summary, while not a traditional virulence factor, iron acquisition is essential to the pathogenesis of *N. gonorrhoeae*, and several redundant mechanisms are present to ensure an adequate supply of iron can be obtained.

### ***Exploitation and evasion of the immune response***

In addition to exploiting host iron-binding proteins for its own benefit, the gonococcus uses host protective mechanisms to its advantage. The complement system is composed of serum proteins that are activated during infection and are an important first line of host defense. As such, *N. gonorrhoeae* has evolved mechanisms to avoid complement-mediated killing. Some strains are inherently serum-resistant (SR) due to binding of host regulatory proteins C4b-binding protein (C4BP) or factor H (fH) to the bacterial porin which causes down-regulation of complement activation (144, 146). Other strains exhibit unstable serum resistance due to a sialyltransferase that catalyzes the addition of host sialic acid to some LOS species and thus masks the bacteria from the immune response and prevents complement deposition (169). Serum-sensitive (SS) strains do not bind C4BP or fH, but some SS strains can use sialylation to protect against complement deposition.



In addition to commandeering host molecules, *Neisseria* spp. have evolved mechanisms to evade antimicrobial peptides and killing by PMNs. Several active efflux pump systems are expressed by the gonococcus (163) and recently, our laboratory showed that the MtrC-MtrD-MtrE pump provides a strong fitness advantage to gonococci in the lower genital tract of female mice (94). Efflux pumps, in general, protect bacteria from harmful substances and the efflux pumps of *N. gonorrhoeae* have been implicated in resistance to macrolides and penicillin, antimicrobial peptides, long chain fatty acids, bile salts, and progesterone. Mutations in the *mtrR* repressor gene or its promoter region are commonly found in clinical isolates and strains with increased pump expression have a fitness advantage during competitive infection with wild type gonococci in the murine lower genital tract (190). During symptomatic infections, a strong inflammatory response characterized by a robust PMN influx is induced. However, recent evidence suggests that PMNs do not kill the gonococcus and that bacteria are capable of replicating within this phagocytic cell (40, 41, 166, 171, 195). Sialylation, in addition to mediating unstable serum resistance, also prevents killing by PMNs as sialylated gonococci are not phagocytosed efficiently and when phagocytosed are less sensitive to killing than non-sialylated bacteria (62, 194). These studies demonstrate that *N. gonorrhoeae* is able to persist, even in the presence of an innate immune response.

As evidenced by the high incidence of reinfection with the same strain or serotype, a protective adaptive immune response is not generated after natural infection (54, 57, 84). As discussed above, there are many redundant and variable surface molecules expressed by *N. gonorrhoeae*, which may prevent the development of an effective immune response to this pathogen. Additionally, the adaptive immune response

may be suppressed by down regulation of lymphocyte activation (23, 135), and hindered by degradation of IgA1 by a secreted IgA protease (138, 139), generation of blocking antibodies against the reduction modifiable protein (Rmp) (150), and molecular mimicry by the addition of host sialic acid to the surface of some LOS variants (118). By utilizing these multiple evasion mechanisms the gonococcus can avoid an effective adaptive immune response.

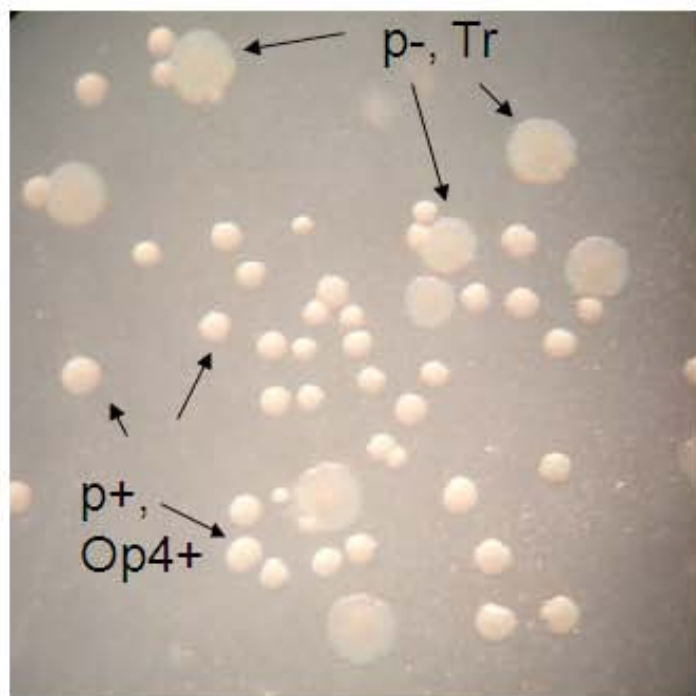
In summary, *N. gonorrhoeae* is a pathogen that has been fine tuned by years of co-evolution with humans. As such, the pathogenesis of this bacterium has adapted whereby many mechanisms are in place to ensure successful colonization and transmission to a new host. Consistent with this evolution of redundant factors, there are up to 11 antigenically distinct Opa proteins expressed by the gonococcus; the role of Opa proteins in establishing and maintaining a productive infection is described below.

### *Opacity protein structure and function*

The Opa proteins are a well characterized family of adhesins that are expressed by the gonococcus and the meningococcus, as well as commensal *Neisseria* spp. Opa proteins were first recognized because their expression confers a characteristic opacity to colonies when viewed under a stereomicroscope (174). It is the interaction between the LOS and Opa proteins within a colony that confers different photo-opacities (15), although not all Opa-expressing gonococci are opaque. The most transparent and opaque variants of strain FA1090 are shown in Figure 2.

**Figure 2. An example of transparent (Opa-negative) and highly opaque (OpaI-expressing) variants of strain FA1090.** Shown are colonies obtained from the lower genital tract of estradiol-treated female mice after 48 hours of incubation on GC agar and are shown as viewed under a stereomicroscope. The most opaque variant (Op4<sup>+</sup>) of strain FA1090 is the OpaI variant and is indicated by arrows. Transparent (Tr) colonies that are Opa-negative are also indicated by arrows. Piliation can also be predicted by colony morphology as piliated colonies are smaller, have a domed morphology, and have a more defined outer edge than do non-piliated colonies. In this figure, all of the Op4<sup>+</sup> variants are piliated (p<sup>+</sup>) and all of the Opa-negative variants are non-piliated (p<sup>-</sup>), although piliation is not linked to Opa expression.

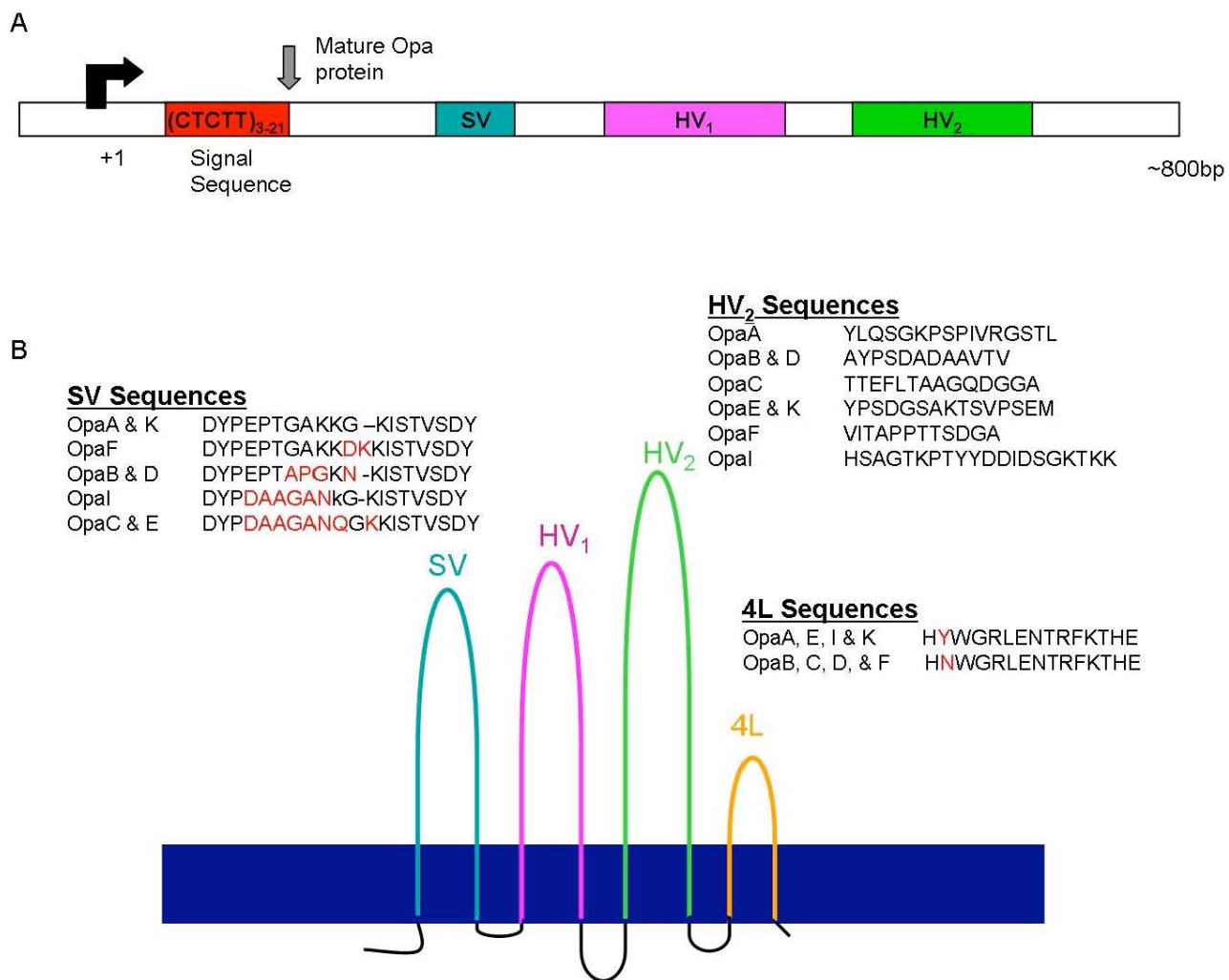
Figure 2. An example of transparent (Opa-negative) and highly opaque (OpaI-expressing) variants of strain FA1090.



All *Neisseria* spp. have *opa* genes, however there are differences between gonococcal, meningococcal, and commensal Opa repertoires. Gonococcal strains carry 11-12 *opa* genes (14, 47), meningococcal strains have 3-4 *opa* genes (170), and the commensal *Neisseria* spp. have 1-2 *opa* genes. Amino acid alignments demonstrate that most of the commensal Opa proteins are distinct and cluster away from the Opa proteins of the pathogenic species (180). These results suggest that while Opa proteins are conserved, their function may differ between pathogenic and commensal *Neisseria* spp..

Each gonococcal or meningococcal strain encodes its own repertoire of Opa proteins, thus most laboratories work only with a single strain when studying Opa protein function. *N. gonorrhoeae* strain FA1090 has 11 *opa* genes (*opaA-K*), which are scattered throughout the chromosome. Eight distinct Opa proteins are expressed by this strain: OpaA, OpaB, OpaC, OpaD, OpaE, OpaF, OpaI, and OpaK (47). The same protein is encoded by the *opaB* and *opaG* loci and the *opaF* and *opaH* loci, while the *opaJ* locus is predicted to be inactive (37, 47, 92). The structure of an *opa* gene is shown in Figure 3A and the backbone of each gene is conserved with three variable regions: a semi-variable (SV) region near the 5' end of the gene and two hypervariable regions (HV<sub>1</sub> and HV<sub>2</sub>) further downstream. Each *opa* gene is independently phase-variable due to frame-shift events that occur in a pentameric (CTCTT) repeat region of the gene (13, 130). During replication of the genome, loss or gain of a single repeat unit causes changes in the downstream reading frame, which results in production of a transcript with an early stop codon, and thus prevents translation of a complete Opa protein. While the phase variation events occur during replication, a change is not apparent until translation. This phase variation event is reversible and it is estimated to occur at a rate of

**Figure 3. Schematic of an *opa* gene and a mature Opa protein.** (A) Representative structure of an *opa* gene with the repeat region (CTCTT) and variable regions indicated. The first variable region is semi-variable (SV), followed by two hypervariable regions (HV<sub>1</sub> and HV<sub>2</sub>). The transcription start site is indicated by a black arrow and the start of a mature Opa protein is indicated by a grey arrow. (B) Predicted structure of an Opa protein with four surface-exposed loops as proposed in (117). The variable regions SV, HV<sub>1</sub> and HV<sub>2</sub> encode for the first three surface-exposed loops and a fourth, highly conserved loop is also present. The predicted amino acid sequences for the SV, HV<sub>2</sub> and 4L of strain FA1090 are shown with divergent amino acids in the SV and 4L sequences indicated in red.

Figure 3. Schematic of an *opa* gene and a mature Opa protein.

$10^{-3}$ /cell/generation (119). There are no known regulators of *opa* gene phase variation, although promoter strength has been shown to influence the rate of phase variation (12). Opa proteins are the second most abundant protein in the outer membrane and have slight differences in molecular weight due to variable regions present in *opa* genes. Opa proteins are expressed as trimers or tetramers in the outer membrane (2), and each individual Opa protein is predicted to have four surface-exposed loops (117). Three of the four loops correspond to the variable regions described above and are depicted in Figure 3B. The fourth loop (4L) is highly conserved with only a single amino acid difference in the eight Opa proteins of strain FA1090, while the first loop is semi-variable (SV) with 67-100% amino acid identity among the Opa proteins of strain FA1090. The second and third loops are the hypervariable loops (HV<sub>1</sub> and HV<sub>2</sub>) and are the longest and most variable loops with only 24-43% identity in the HV<sub>2</sub> loops of strain FA1090 Opa variants. The HV<sub>2</sub> loop is the immunodominant region of the protein (44), and thus, an immune response directed at a mature Opa protein during infection is not likely to be protective against heterologous Opa variants.

Tissue culture studies have clearly demonstrated that Opa proteins interact with several host receptors. Most Opa proteins studied to date interact with members of the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family, namely CEACAM1, CEACAM3, CEACAM5 or CEACAM6 (18, 30, 67, 68, 187). CEACAMs belong to the immunoglobulin superfamily of cell adhesion molecules and can be associated with the cellular membrane through either a glycosylphosphatidylinositol (GPI) anchor or a cytoplasmic domain. Members of the CEACAM family have a wide



tissue distribution including epithelial, endothelial, and immune cells, and have important functions in diverse cellular processes (reviewed in (105)).

Opa-CEACAM interactions promote bacterial invasion of the host cell and these interactions require both HV<sub>1</sub> and HV<sub>2</sub> loop sequences (21). All Opa-CEACAM interactions occur through binding to conserved residues in the N-terminal region of the immunoglobulin variable-like domain of CEACAMs (19, 142, 188). The signal transduction pathways that are induced after receptor binding differ between cell types depending on the CEACAM that is expressed. Signaling via the granulocyte-specific CEACAM3 requires the Src family of protein tyrosine kinases and epithelial cell CEACAM1, CEACAM5, and CEACAM6 utilize lipid raft-mediated uptake of gonococci (158). Since the identification of CEACAMs as receptors for Opa proteins, it has been shown that the outer membrane proteins UspA of *Moraxella catarrhalis* and P5 of *Haemophilus influenzae* also bind CEACAM1, despite being structurally unrelated bacterial proteins (17, 80, 81). Thus, several mucosal pathogens exploit this family of host receptors.

In addition to promoting invasion into host cells, Opa-CEACAM interactions may have immunomodulatory roles, which are mediated through the interactions of specific kinases with the transmembrane domain of CEACAM1. Interaction between Opa proteins and CEACAM1 on primary CD4<sup>+</sup> T lymphocytes resulted in a decrease in lymphocyte activation as measured by CD69 expression and cellular proliferation (23). This interaction was independent of bacterial uptake and did not require bacterial replication, as outer membrane vesicles from Opa-positive gonococci were also capable of decreasing CD4<sup>+</sup> lymphocyte function (110, 111). B lymphocytes also express

CEACAM1, and Opa-CEACAM interactions on B cells cause a decrease in antibody production and an increase in cell death without bacterial internalization (135). CD4<sup>+</sup> cells are inhibited through the actions of the tyrosine phosphatases SHP1 and SHP2 on immunoreceptor tyrosine-based inhibition motifs (ITIMs), while the effects on B cells are ITIM-independent and instead utilize Bruton's tyrosine kinase (23, 135). Recently, an immunosuppressive effect of *N. meningitidis* Opa-CEACAM1 interaction on pulmonary epithelial cells was shown. CEACAM-ligation by Opa-expressing *Escherichia coli* inhibits TLR2-dependent activation of primary bronchial epithelial cells as measured by decreased IL-8 release. This effect is mediated by interaction of ITIMs with SHP-1 (168), similar to what is seen with CD4<sup>+</sup> lymphocytes. Immunosuppression of the innate and adaptive immune response via Opa-CEACAM1 interactions may contribute to the ability of this pathogen to evade clearance by the host.

Some Opa proteins interact with heparin sulfate proteoglycans (HSPGs) (29, 183) and through the use of chimeric Opa proteins from *N. meningitidis*, the HV<sub>1</sub> loop was implicated in HSPG-binding (66). While interaction with HSPG allows for bacterial adherence, invasion requires a coordinate interaction between HSPG and the syndecans vitronectin and/or fibronectin to promote invasion of adherent gonococci (50, 63, 182). Thus, Opa proteins have been shown to mediate adherence and invasion of tissue culture cells through several different mechanisms, although most Opa proteins preferentially bind to CEACAMs.

Most studies on Opa proteins have examined their role in adherence and invasion or immunosuppression of host cells. However, a single study with *N. gonorrhoeae* strain MS11 demonstrated that Opa-positive bacteria are more resistant to the bactericidal

activity of low concentrations of normal human sera (NHS) than Opa-negative bacteria (20). This result suggests Opa-positive gonococci are not as susceptible to complement-mediated killing on mucosal surfaces. In summary, as described here, several potential roles for Opa proteins have been predicted in the literature based on *in vitro* analyses.

### *Challenges in Neisseria gonorrhoeae vaccine development*

Control of gonorrhea has been greatly improved by the discovery and development of antibiotics. In the 1930s, sulfanilamide was used to treat gonorrhea and was followed closely by the clinical use of penicillin in the 1940s. Resistance to antibiotics evolved as new drugs were introduced throughout the latter part of the 20<sup>th</sup> century (87). Currently, the cephalosporins are the only class of antibiotics recommended for treatment of *N. gonorrhoeae* in the US (1), and resistance to this last line of drugs has already been observed (114). As our antibiotic options become limited, the need for a gonococcal vaccine becomes urgent.

### *Lack of clear protective correlates*

The highly variable nature of the gonococcus and the lack of clear correlates of protection have challenged vaccine development. While there is no gonococcal vaccine available, there are several meningococcal vaccines currently licensed. The vast majority of these vaccines are based upon the capsular type, which is an essential virulence factor during invasive meningococcal infections, but is not expressed by the gonococcus (26). Gonococcal vaccine development has been influenced by the demonstration that a humoral response is protective against *N. meningitidis* and that, specifically, meningococcal vaccines that induce antibodies with bactericidal activity or

opsonophagocytic activity correlate with protection (65). However, while bactericidal antibodies are important for protection against invasive meningococcal disease, naturally acquired bactericidal antibodies against the gonococcus do not correlate with disease clearance (100). As an additional challenge, antibodies need to be bactericidal against SR gonococcal strains, and some gonococcal strains are naturally resistant to up to 50% NHS.

As natural immunity does not develop during infection, there is limited information available on what is protective against gonorrhea. Gonococcal vaccines based on porin, pili, Opa, the transferrin receptor, and outer membrane vesicles have all been characterized for the generation of antibodies with bactericidal activity, opsonophagocytic capacity, and/or the ability to block interactions with tissue culture cells (32, 38, 43, 44, 152, 197). While most vaccines have not been tested *in vivo*, a pilin-based vaccine was assessed in human phase I/II trials. No protection against gonorrhea was seen in vaccinated individuals compared with unvaccinated controls (22).

### ***Passive vaccination with antibodies against Opa proteins***

We are interested in investigating the potential of passive protection as an approach to gonococcal vaccine development. There has been much interest recently in providing affordable passive vaccination for STIs that can be combined with contraceptives for high risk populations (89). Antibody administration was effective at preventing infection with HSV-2 and *Chlamydia* (134, 193), and antibodies could be combined with spermicides to prevent transmission of STIs and provide concurrent, affordable contraception for women. As *N. gonorrhoeae* is thought to be a primarily

extracellular pathogen, the presence of antibodies at mucosal sites will presumably be an important component of a protective immune response.

Opa proteins undergo phase variable expression; however, there is evidence in support of their potential as a vaccine target. Opa protein-specific antibodies are generated during natural infection, presumably to the HV regions, with detectable antibodies in serum and genital secretions from men and women with uncomplicated urogenital tract infections, PID, or DGI (109, 196). Additionally, the presence of antibodies to multiple Opa proteins is associated with a reduced risk of PID in commercial sex workers (140). Vaccination of mice with purified meningococcal Opa proteins, or Opa proteins expressed in liposomes, demonstrated that the HV<sub>2</sub> loop is the immunodominant region of the protein (43, 44). These studies suggest that while antibodies are elicited against the HV regions, they are not protective against genital tract disease. We believe that Opa proteins may be effective vaccine antigens if antibodies are generated against conserved regions of the protein. The recent development of an animal model (91) should allow a better understanding of the *in vitro* correlates of protection for gonorrhea.

### ***Animal models***

While the gonococcus has been extensively studied *in vitro*, *in vivo* studies are limited because *N. gonorrhoeae* is a human-specific pathogen. The use of subcutaneous chambers in mice and guinea pigs has provided some information on virulence factors (8, 28, 60, 120), but the results are limited in application as these models do not accurately reflect the site of infection or route of inoculation. Non-human primates (chimpanzees)

have been used for studies of genital tract disease and transmission but are impractical due to the expense and the scarcity of animals available for studies (8-10).

Experimental infection of 17 $\beta$ -estradiol-treated female mice closely mimics many of the characteristics of human infection and therefore should be useful for testing the efficacy of gonococcal vaccines. We recently characterized the immune response in infected, estradiol-treated mice and found that the humoral immune response is transient and does not protect against reinfection (172), which is similar to what is observed clinically in patients with gonorrhea (78, 124, 140). To date, our laboratory has tested several vaccine antigens but, unfortunately, none have showed protection against experimental murine infection. Use of the mouse model to test candidate vaccines should allow us to define correlates of protection for *N. gonorrhoeae* and facilitate screening of potential vaccine antigens.

### *Role of Opa proteins during infection*

The importance of Opa proteins *in vivo* has been studied in two experimental systems: intraurethral inoculation of male volunteers (92, 157, 176) and intravaginal inoculation of estradiol-treated mice (91, 165). In human studies, male volunteers were inoculated intraurethrally with predominantly piliated, Opa-negative variants of either strain FA1090 or strain MS11 and the recovery of gonococci was measured in urine and urethral discharge by quantitative culture. Men were treated with antibiotics to clear the infection when urethral discharge was observed or after 5 days of infection (34). After inoculation of men with predominantly Opa-negative variants, most gonococci were Opa-positive in the first positive urine culture (92, 157, 176). In studies with strain FA1090,

no one Opa protein seemed to predominate in all subjects. However within a subject, repeated samples showed expression of the same Opa protein for up to 5 days (92). Similar results were obtained with strain MS11 (157, 176). These data strongly suggest that in the male urethra Opa proteins are highly advantageous and results are consistent with the recovery of predominantly Opa-positive variants from urethral isolates obtained from naturally infected men (90). Despite this strong selection for Opa-positive variants in wild-type infections, inoculation with a genetically modified strain of FA1090 that is deficient for Opa expression infected volunteers for 5 days and caused a urethral discharge that was grossly indistinguishable from that seen with wild type infections (58). These results suggest that while Opa protein expression is selected for, it is not required for infection of the male urethra.

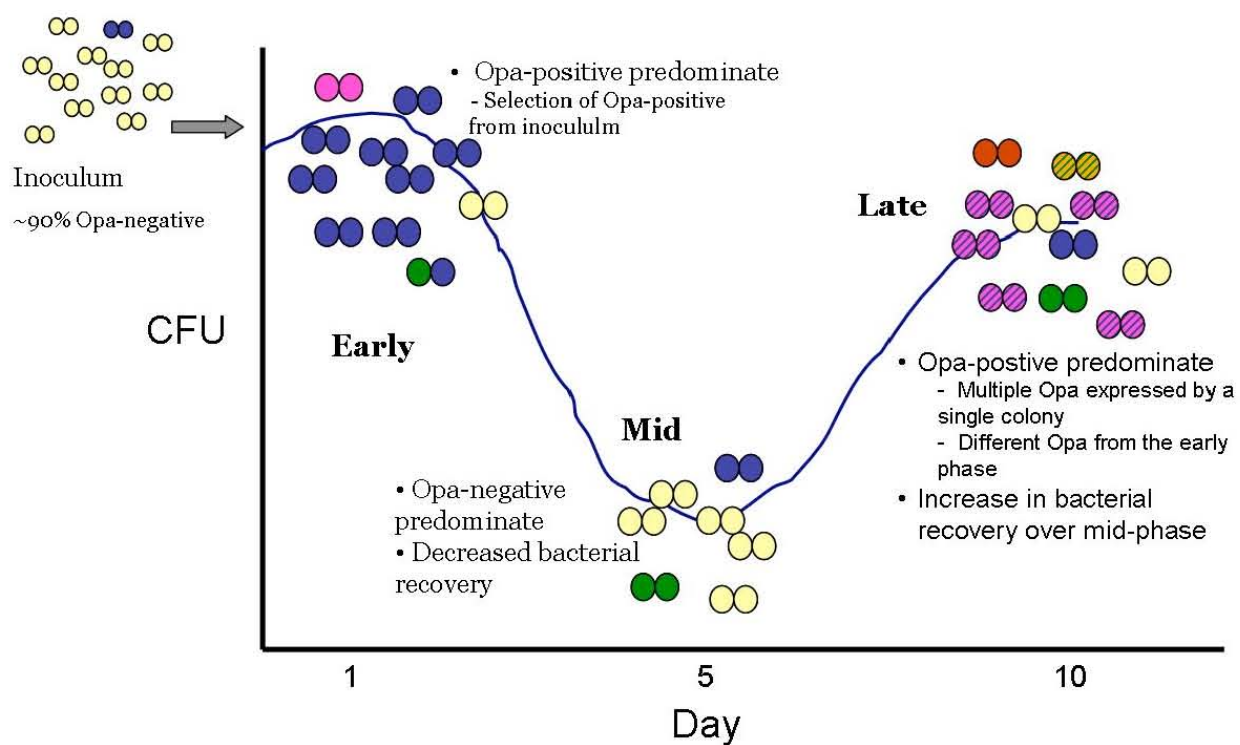
There appears to be a selective advantage for Opa expression in the male urethra, but the reason for this advantage is unknown. Analysis of primary male urethral epithelial cells, which are the main cells infected during uncomplicated urethral infection, demonstrated only limited CEACAM3 expression, and no detection of the other CEACAMs (75). The advantage seen for Opa-positive gonococci in male volunteers may, therefore, not simply be due to better bacterial adherence or invasion, but may be due to Opa-mediated resistance to innate factors.

A second *in vivo* model was developed in our laboratory and utilizes estradiol treatment of female mice to promote long-term lower genital tract infection (91, 172). We recently showed that infection of the murine genital tract with predominantly Opa-

**Figure 4.** Mice treated with a 5 mg slow-release  $17\beta$ -estradiol pellet show cyclical recovery of Opa-positive variants and fluctuations in bacterial load. Previous studies by our laboratory demonstrated that recovery of Opa variants from the lower genital tract of estradiol-treated mice occurs in three distinct phases. These phases are indicated in the figure and the Opa phenotype (circles) and CFU (line) recovered from mice in the early, mid and late phases are shown. (Adapted from (165).)



Figure 4. Mice treated with a 5 mg slow-release 17 $\beta$ -estradiol pellet show cyclical recovery of Opa-positive variants and fluctuations in bacterial load.



negative variants resulted in cyclical recovery of Opa-positive variants, as well as fluctuations in the number of total bacteria recovered (165). We identified three distinct phases of recovery for Opa variants in the mouse model which are illustrated in Figure 4. Following inoculation of mice with a predominantly Opa-negative inoculum, Opa-expressing gonococci were selected from the inoculum (early phase). Following the early phase, there was a decreased recovery of Opa-positive variants with a corresponding decrease in the overall number of bacteria recovered by vaginal swab (mid-phase). Following the mid-phase is the late phase in which an increase in the recovery of Opa-positive isolates and an increase in the number of CFU recovered occurred. The late phase is also characterized by a predominance of gonococci that expressed multiple Opa proteins, which was also seen in cultures obtained at late time points from experimentally infected men (92, 157, 176).

The basis for the cyclical recovery pattern of Opa variants from female mice is unknown. Mice express a single CEACAM; however, comparison of the human and murine CEACAM1 predicted amino acid sequences suggest murine CEACAM1 does not support Opa binding due to differences in several critical amino acids (185, 186). Our characterization of Opa selection during murine infection suggests there is a separate, CEACAM-independent function of Opa proteins as Opa-positive gonococci had an advantage in a model that lacks human CEACAMs (165). These results bring into question the clinical importance of CEACAMs during lower urogenital tract infection of women and indicate that another function for Opa proteins, perhaps complement resistance, may be present in the female lower genital tract.

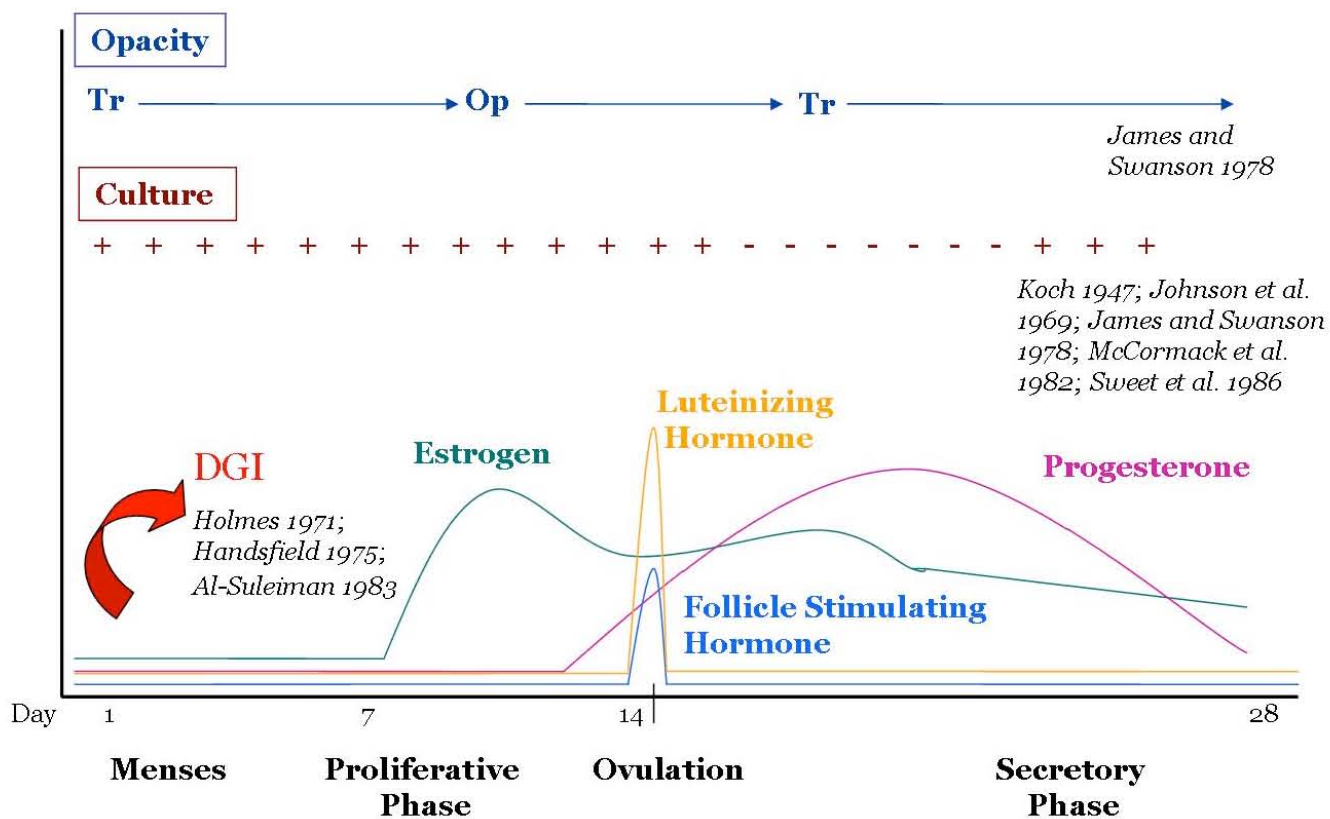
### *Influence of the reproductive cycle on gonorrhea*

The female reproductive cycle is a tightly controlled set of events that prepare the endometrium for implantation and averages 28 days in most women. The stages of the menstrual cycle and the hormones involved are diagramed in Figure 5. Following menstruation (days 1-5), estradiol is secreted in increasing amounts by the ovaries and promotes proliferation of the endometrium in preparation for ovulation. The proliferative phase is approximately two weeks in length and ends with ovulation, which is triggered by a surge in luteinizing hormone (LH) and follicle stimulating hormone (FSH) produced from the anterior pituitary gland. The secretory phase follows ovulation and is characterized by high levels of estradiol and progesterone. During the secretory phase the endometrium is ready for implantation, but if implantation does not occur, the levels of estradiol and progesterone decrease and menstruation begins (71).

Many studies suggest gonorrhea is modulated by the female reproductive cycle. Prepubescent girls and post-menopausal women commonly present with vaginitis, while menstruating women present with cervicitis (70). In a 10 year retrospective study of patients in Seattle at 3 different public and private health care settings, there were 42 cases of DGI, 33 (78%) of which were in women. These results suggest women are more likely than men to present with DGI. Of the 33 female patients, 9 (27%) were pregnant or had delivered within two weeks, and 14 (42%) were in the first 7 days of their menstrual cycle. Thirteen of fourteen women who presented with DGI in the first 7 days of their cycle were menstruating (85). These results are not predicted from a normal distribution of DGI throughout the menstrual cycle, and suggest dissemination is more likely during menstruation. Similar results have been observed in several studies (5)

**Figure 5. Correlation between the menstrual cycle, DGI, culture positivity, and recovery of Opa phenotypes from women.** Several studies report a link between the menstrual cycle and DGI, as well as the overall culture positivity and colony opacity of cervical isolates. These results are summarized here, with the relative levels of hormones during the reproductive cycle indicated and the culture positivity and Opa phenotype of cervical isolates summarized above. Isolates obtained near menstruation were likely to be transparent (Tr) and those isolates obtained near ovulation were likely to be opaque (Op). Following ovulation there is a decrease in the recovery of gonococci from infected women, and dissemination occurs most often during or near menstruation.

Figure 5. Correlation between the menstrual cycle, DGI, culture positivity, and recovery of Opa phenotypes from women



(74). These clinical observations provide evidence for a link between gonococcal infection and the female reproductive cycle.

The molecular basis for the increased dissemination near menstruation is not known and is challenged by the lack of a DGI model that starts with a mucosal infection. Primates are the only species that menstruate and no studies have been done to address DGI in non-human primates. In correlation with DGI cases being more common around menstruation, isolates from women at or near menstruation were more likely to express the hemoglobin receptor as compared to women at later times in the menstrual cycle (7) as discussed previously. The presence of hemoglobin may allow for better bacterial replication and potentially higher numbers of invasive bacteria. Opa phenotype also seems to be important during dissemination as over 90% of blood isolates were transparent (Opa-negative) in a single study of 49 patients with DGI (133).

While no animal model exists to address the role of hormones and bacterial factors in susceptibility to DGI, fallopian tube organ cultures (FTOC) have been utilized to investigate potential factors involved in gonococcal ascension into the upper reproductive tract and invasion across the epithelium (64, 116, 123). Opa proteins that were constitutively expressed in *E. coli* mediated increased adherence and invasion into non-ciliated cells of FTOCs. However, porin, but not Opa expression, promoted increased invasion into the subepithelial space (64). Thus, Opa expression could promote ascension from the cervix to the upper reproductive tract, where internalized, Opa-negative bacteria can then cross the mucosa to cause invasive disease. An appropriate animal model is needed to test factors implicated by these studies.

The Opa phenotype of cervical isolates also appears to be linked to the hormonal status of women. Studies on gonococcal infection in women are restricted to the analysis of clinical isolates due to the risk of severe complications in female volunteers. In a study of 104 cervical isolates from naturally infected women, culture positivity and presence of trypsin-resistant (Opa-negative) or trypsin-sensitive (Opa-positive) variants were analyzed with respect to the stage of the menstrual cycle. During menstruation women were most likely to be culture positive and colonized with transparent, trypsin-resistant (Opa-negative) variants. Near ovulation most gonococci were opaque and trypsin sensitive (Opa-positive) and the lowest recovery of gonococci occurred during the secretory phase of the menstrual cycle. Women taking oral contraceptives showed similar correlations between the menstrual cycle and the trypsin sensitivity phenotype, but the degree of variation in Opa phenotype was not as dramatic (90). The link between recovery of gonococci and the menstrual cycle has been supported in several other studies of naturally infected women (96, 104, 121, 178). These studies strongly suggest a hormonally-linked factor influences the recovery of Opa variants and several potential factors are listed in Figure 20. A summary of these results with respect to the menstrual cycle is shown in Figure 5.

### *Thesis Objectives*

There are two main objectives of this dissertation: to investigate the potential of conserved Opa protein loops as targets for passive vaccination and to investigate the importance of Opa proteins during infection of the murine female genital tract.

My first objective is presented in Chapter 2 and was undertaken to characterize the potential of conserved Opa loops as targets for passive protection. Opa proteins contain conserved regions and are commonly expressed during genital tract infections of men and women. We hypothesized that antibodies that target conserved loops will bind multiple Opa variants and can be used to protect against infection with gonococci that express different Opa proteins. To this end, we tested both linear and cyclic peptide-derived antibodies against the two conserved Opa loops for the capacity to bind Opa variants, and we used *in vitro* assays and *in vivo* studies to begin to define correlates of protection for *N. gonorrhoeae*.

My second objective was to further characterize the cyclical recovery pattern of Opa variants previously observed in female mice and to investigate the importance of Opa expression during infection. Opa proteins provide an advantage during lower genital tract infection of men and female mice and have been implicated in adherence and invasion of host cells, evasion of innate factors, and immunosuppression of the host. While Opa proteins are hypothesized to be important during infection, their expression during urethral infection of men is not essential. In females, Opa protein expression appears to be linked to the reproductive cycle, although the molecular basis for this link is unknown.

We hypothesized that the phase variable expression of *opa* genes allows the gonococcus to evade hormonally-regulated factors in the female reproductive tract. To address this hypothesis, we characterized the cyclical recovery pattern of Opa variants in ovariectomized mice and investigated the role of Opa-mediated adherence and complement in mediating the cyclical recovery pattern. We also investigated the capacity



of an Opa-deficient strain to colonize female mice, compared to a strain that constitutively expresses a single Opa protein. This work is presented in Chapter 3.

## Chapter 2: Functional Characterization of Antibodies against *Neisseria gonorrhoeae* Opacity Protein Loops

Jessica G. Cole and Ann E. Jerse

### *Abstract*

The development of a gonorrhea vaccine is challenged by the lack of correlates of protection. The antigenically variable neisserial opacity (Opa) proteins are expressed during infection and have a semivariable (SV) and highly conserved (4L) loop that could be targeted in a vaccine. Here we compared antibodies to linear (Ab<sub>linear</sub>) and cyclic (Ab<sub>cyclic</sub>) peptides that correspond to the SV and 4L loops and selected hypervariable (HV<sub>2</sub>) loops for surface-binding and protective activity *in vitro* and *in vivo*. Ab<sub>SV cyclic</sub> bound a greater number of different Opa variants than did Ab<sub>SV linear</sub>, including variants that differed by seven amino acids. Antibodies to the 4L peptide did not bind Opa-expressing bacteria. Ab<sub>SV cyclic</sub> and Ab<sub>HV2 cyclic</sub>, but not Ab<sub>SV linear</sub> or Ab<sub>HV2 linear</sub> agglutinated homologous Opa variants, and Ab<sub>HV2BD cyclic</sub> but not Ab<sub>HV2BD linear</sub> blocked the association of OpaB variants with human endocervical cells. Only Ab<sub>HV2BD linear</sub> were bactericidal against the serum resistant parent strain. Consistent with host restrictions in the complement cascade, the bactericidal activity of Ab<sub>HV2BD linear</sub> was increased 8-fold when rabbit complement was used. None of the antibodies was protective when administered vaginally to mice. Antibody duration in the vagina was short-lived, however, with < 50% of the antibodies recovered 3 hrs post-administration. We conclude that an SV loop-specific cyclic peptide can be used to induce antibodies that recognize a

broad spectrum of antigenically distinct Opa variants and have agglutination abilities. HV<sub>2</sub> loop-specific cyclic peptides elicited antibodies with agglutination and adherence blocking abilities. The use of human complement when testing the bactericidal activity of vaccine-induced antibodies against serum resistant gonococci is also important.

*Note: All of the experimentation in this study was performed by J.G. Cole except for technical assistance as indicated in the acknowledgements. At the time of this writing, this manuscript was accepted in PlosOne and is being revised based on reviewers' comments.*

### *Introduction*

Gonorrhea is the second most commonly reported disease in the United States with over 350,000 cases reported in 2006 (125) and over 62 million estimated annual cases worldwide (61). The gonococcus colonizes many mucosal sites, including the cervix, urethra, rectum, and pharynx. Ascended reproductive tract infections are the major source of the morbidity and mortality associated with this pathogen. Ascended infection occurs in 10-20% of cervical infections, and can lead to pelvic inflammatory disease (PID) and the associated complications of involuntary infertility, ectopic pregnancy, and chronic pelvic pain (87). Gonorrhea is also a co-factor for transmission of the human immunodeficiency virus (36). The public health cost of gonococcal infections is significant; over 77 million dollars were spent in the U.S. in the year 2000 on the diagnosis and treatment of acute gonorrhea and post-infection sequelae in patients 15-24 years of age (31). The relatively rapid emergence of antibiotic-resistant strains (179), is

illustrated by the recent removal of fluoroquinolones from recommended treatments (1), and underscores the importance of identifying new preventive measures against gonorrhea.

The development of a gonorrhea vaccine is challenged by the lack of known correlates of protection. Repeat infections are common, even with the homologous strain (54) or serotype (57, 84), although evidence of partial immunity has been reported (27, 141). *N. gonorrhoeae* does not express a capsule, which is the target of several effective meningococcal vaccines. Therefore, research towards a gonorrhea vaccine has focused on other surface antigens such as outer membrane proteins. The neisserial opacity (Opa) proteins are a family of outer membrane proteins that mediate adherence to and invasion of tissue culture cells (46). Gonococcal strains carry up to 11 *opa* genes (14, 47) that encode 8-11 antigenically distinct proteins. Mature Opa proteins are predicted to have four surface-exposed loops, namely, one semi-variable (SV) loop, two hypervariable (HV<sub>1</sub> and HV<sub>2</sub>) loops, and one conserved (4L) loop (117). Sequence differences in the HV regions are responsible for the antigenic identity of each Opa protein as well as slight differences in molecular weight. Each *opa* gene undergoes phase variation via a frame shift mechanism, and therefore, a single gonococcus can express no Opa proteins, one Opa protein, or multiple Opa proteins simultaneously (130, 173).

The expression of Opa proteins by *N. gonorrhoeae* appears to be important during urogenital tract infections. The majority of urethral isolates from naturally (90) and experimentally infected men (92, 176) expressed one or more Opa proteins, and in women, mostly Opa-positive isolates were recovered from the cervix during certain stages of the menstrual cycle (90). Evidence for Opa protein expression during infection

is also supported by the detection of Opa protein-specific antibodies in serum and genital secretions from men and women with uncomplicated urogenital tract infections, PID, or disseminated gonococcal infection (109, 196). The presence of antibodies to multiple Opa proteins is associated with a reduced risk of PID in commercial sex workers (140), and therefore, Opa proteins may be protective vaccine antigens.

While the HV loops are highly variable among Opa proteins, the SV and 4L loops are relatively and highly conserved, respectively. Therefore, the SV and 4L loops might be used to generate broadly reactive antibodies that bind many different Opa variants. A caveat to immunization with whole Opa proteins is that the HV loops are immunodominant and may prevent generation of high levels of antibodies against more conserved regions of the protein (44). Additionally, Opa-mediated interactions with CEACAM1 on B and T lymphocytes may decrease the effector functions of these immune cells, and thus prevent a robust vaccine-induced immune response (23, 135), although this hypothesis was not supported by studies with meningococcal Opa-specific antibodies (43). To avoid these potential pitfalls and to test whether the SV and 4L loops might carry broadly reactive, protective epitopes, here we utilize peptide-based immunization strategies to generate antibodies against the SV and 4L loops. We tested Opa loop-specific antibodies for specificity, surface-binding, agglutination, and bactericidal activities. Antibodies against peptides that correspond to certain HV<sub>2</sub>-loops were tested in comparison. Both linear and cyclic peptides were used to generate antibodies based on evidence that cyclic peptides induced bactericidal, conformation-dependant antibodies against meningococcal outer membrane proteins (32). Finally, we also investigated the capacity of Opa loop-specific antibodies to inhibit gonococcal

adherence to human tissue culture cells and to protect female mice from experimental genital tract infection when delivered topically.

### *Materials and Methods*

***Strains and culture conditions*** *Neisseria gonorrhoeae* strain FA1090 [*porB1b*, streptomycin resistant, serum resistant (SR)] was originally isolated from a female patient with disseminated gonococcal infection. Strain FA1090 expresses 8 antigenically distinct Opa proteins: OpaA, OpaB, OpaC, OpaD, OpaE, OpaF, OpaI and OpaK. Frozen stocks of each Opa variant were prepared as described (165). Stock Opa variants expressed LOS species with the same banding pattern on silver stained tricine gels (data not shown) and stocks composed of either mostly piliated or nonpiliated variants were maintained. Strain FA1090<sub>F62por5-8</sub> is a serum sensitive (SS) derivative of strain FA1090 in which porin loops 5-8 were replaced with loops 5-8 of the SS strain F62 as described by Ram et al. (kindly provided by Sanjay Ram, University of Massachusetts) (144). Strain FA1090<sub>F62por5-8</sub> is sensitive to NHS, and does not bind human C4b-binding protein (hC4BP). OpaA, OpaF, and Opa-negative variants were isolated from OpaB-expressing FA1090<sub>F62por5-8</sub> bacteria after 2-3 serial passages of individual colonies that were screened by colony suspension immunoblots with HV<sub>2</sub>-specific antibodies as described (91, 165). Where indicated, recombinant strains of FA1090 that express no Opa proteins or that constitutively express only OpaB were used (kindly provided by Janne Cannon, University of North Carolina). *N. gonorrhoeae* was cultured on GC agar (Difco) with Kellogg's supplement (101) and 0.2  $\mu$ M Fe(NO<sub>3</sub>)<sub>3</sub> at 37°C under 7% CO<sub>2</sub>. GC-VCNTS

agar (GC agar with vancomycin, colistin, nystatin, trimethoprim, and streptomycin sulfate) was as described (91).

***Generation of antibodies*** Affinity-purified rabbit polyclonal antibodies against linear peptides that correspond to the 4L and the HV<sub>2</sub> loop sequences of OpaA, OpaB, OpaC, OpaD, OpaF, OpaI, and OpaK variants were previously described (165). The 4L peptide sequence was HYWGRLNTRFKTHE. Here, affinity-purified rabbit polyclonal SV-loop specific antibodies (Ab<sub>SV linear</sub>) were generated against a linear 20 amino acid peptide (DYPEPTGAKKGKISTVSDYF) that corresponds to the SV loop of OpaA and OpaK (OpaAK) of strain FA1090 (Figure 6). Peptide synthesis, rabbit immunizations, and affinity purification were performed by Bethyl Laboratories (Montgomery, Texas). Rabbit antibodies were dialyzed (50 kDa exclusions pore size) (Spectrum Laboratories Inc, Racho Dominguez, CA) to remove the 0.1% sodium azide that was added during preparation. We also generated mouse antisera to two cyclic peptides that correspond to the SV loop sequence of OpaAK (AAERITHDYPEPTGAKKGKISTVSDYFRNIRTHSIH; 36-mer) or the HV<sub>2</sub> loop sequence that is common to OpaB and OpaD (OpaBD) (IDSTKKITGTLTAYPSDADAAVTVPDGHQPKNQTYQ; 36-mer). Cyclic peptides were synthesized by Celtek Peptides (Nashville, TN) through the addition of a disulfide bond between added terminal cysteine residues. Six week-old female BALB/c mice were immunized subcutaneously three times at three week intervals with 50 µg of peptide suspended in TiterMax Gold (Sigma Chemical Co, St. Louis, MO). Blood was collected two weeks after the final boost, and individual samples were analyzed by enzyme linked

immunosorbent assay (ELISA) for peptide-specific antibody titers essentially as described (45).

Briefly, 96-well plates were coated with 5  $\mu$ g of the cyclic peptide in 50 mM  $\text{NaHCO}_3$  (pH 9.6), and incubated with three-fold dilutions of mouse sera followed by goat anti-mouse IgG ( $\gamma$  chain-specific) conjugated to horseradish peroxidase (HRP) and HRP substrate (Sigma). Absorbance was read at 405 nm on an EL800 Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT) and analyzed with KC Junior software (Bio-Tek Instruments). Background was set at 3 standard deviations above the average  $A_{405}$  readings of 3 wells to which no primary antibody was added. Sera with titers  $>1:7,290$  were pooled and frozen at  $-20^\circ\text{C}$ . The relative levels of IgG isotypes within Opa loop-specific mouse antisera were measured with a mouse antibody isotyping kit (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer's instructions.

***Immunoblots*** For western blots, bacteria were suspended in lithium acetate buffer and incubated in Laemmli buffer (Sigma) containing sodium dodecyl sulfate (SDS) and  $\beta$ -mercaptoethanol for 10 min at  $100^\circ\text{C}$  to denature the samples or at room temperature (RT) to preserved native conformations. Samples were fractionated on 11.5% SDS polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 0.5% Tween 20, membranes were incubated with  $\text{Ab}_{\text{SV linear}}$  (1:75,000),  $\text{Ab}_{4\text{L linear}}$  (1:6,000), or  $\text{Ab}_{\text{SV cyclic}}$  (1:6,000), followed by goat anti-rabbit IgG HRP (1:50,000) (Bethyl Laboratories) or anti-mouse IgG HRP (1:50,000) (Sigma). Primary and secondary antibodies were diluted in block, and blots were washed three times in PBS with 0.05% Tween 20 after each incubation. Detection was with a chemiluminescent detection reagent (Amersham Biosciences) as per the manufacturer's instructions.



A semi-quantative surface-binding immunoblot (SBI) similar to that described by Afonina et al. (4) was used to measure the binding of antibodies to intact gonococci. Bacteria of the Opa phenotype to be tested were suspended in PBS to an  $A_{600}$  of 0.20 and diluted 1:20 in PBS. One hundred microliters ( $\sim 5 \times 10^5$  CFU) of the final suspensions were applied to a nitrocellulose membrane via a 96-well vacuum manifold apparatus (Schleicher & Schuell, Keene, NH). The membrane was dried at RT, incubated for 30 min at  $37^\circ\text{C}$ , and then blocked for 1 hr in PBS with 3% BSA (Sigma). The filter was returned to the manifold and individual wells were incubated for 1 hr with 100  $\mu\text{L}$  of two-fold serial dilutions of affinity-purified rabbit antibodies ( $\text{Ab}_{\text{SV linear}}$ ,  $\text{Ab}_{4\text{L}}$ ,  $\text{Ab}_{\text{HV2 linear}}$ ; range 8.2-720 ng) or mouse antisera ( $\text{Ab}_{\text{SV cyclic}}$ ,  $\text{Ab}_{\text{HV2BD cyclic}}$ ; range 1:20-1:320). Positive control wells were incubated with serial dilutions of rabbit polyclonal antiserum against heat-killed FA1090 bacteria (1:4,000-1:64,000) or the porin-specific monoclonal antibody B2E8 (1:250-1:4,000) (A.E. Jerse and Mary Petzke, unpublished data). All antibodies were diluted in PBS with 3% BSA, and secondary detection, washes, and exposure of the membranes to substrate were as for western blots. Spot intensities were quantified by densitometry (Image J Version 1.37v) and the mean intensity of three wells incubated without primary antibody was subtracted from that of wells with Opa-specific antibodies (test wells) or anti-whole bacteria or B2E8 antibodies (control wells). The spot intensities of the control wells were plotted against the antibody concentration ( $\text{Ab}_{\text{linear}}$ , rabbit) or antiserum dilution ( $\text{Ab}_{\text{cyclic}}$ , mouse), and values that fell within the linear regions of the curves were used to normalize for slight differences in the number of bacteria in each spot. Normalized data were obtained by dividing the mean intensity of the test wells by that of the appropriate control well (mouse or rabbit antibody).

***Indirect fluorescent antibody (IFA) staining*** Single colonies of strain FA1090

Opa variants were suspended in water, applied to IFA slides (Electron Microscopy Sciences, Hatfield, PA), and fixed in 100% methanol at -20°C after drying at RT. Slides were blocked for 1 hr in PBS with 0.1% immunoglobulin-free BSA (Sigma)(blocking buffer). Slides were incubated with primary antibodies for 1 hr at the following final concentrations or dilutions, which were determined empirically: Ab<sub>HV2 linear</sub> (0.87-1.2 µg/mL), Ab<sub>SV linear</sub> (2.2 µg/mL), Ab<sub>4L linear</sub> (2.4 µg/ml), and Ab<sub>HV2 cyclic</sub> (1:100) and Ab<sub>SV cyclic</sub> (1:30). Secondary antibodies were goat anti-rabbit or goat anti-mouse IgG conjugated to AlexaFluor 488 (Invitrogen, Carlsbad, CA) (1:500), and incubations were for 30 min. All antibodies were diluted in blocking buffer and wells were washed five times with PBS after each incubation. Polyclonal rabbit antisera against heat-killed FA1090 (1:1,000) was used as a positive control; negative controls were antibodies against heterologous HV<sub>2</sub> loops and wells that were not incubated with primary antibodies. Slides were examined with an Olympus BX60 system microscope with a BX-FLA reflected light fluorescence attachment and Olympus U-M41001 filter. All images were obtained with a SPOT charge-coupled-device digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

***Bactericidal assay*** Bactericidal assays against wild type FA1090 Opa variants

were performed in microtiter plates using 10% normal human serum (NHS) (Quidel Corporation, San Diego, CA) or 1% baby bunny serum (BBS) (AbD Serotec, Raleigh, NC) as the complement source. For assays that used strain FA1090<sub>F62por5-8</sub>, 1% NHS was used. These serum concentrations are > 2-fold lower than that concentration that showed no killing of the target strains in the absence of added antibody as determined by Garvin

*et al.* (59). For testing Opa loop-specific antibodies, Ab<sub>linear</sub> or Ab<sub>cyclic</sub> were serially diluted in minimal essential medium (MEM) (final volume 150 µl), and 50 µl of diluted NHS or BBS were added to each well to achieve the final serum concentrations stated above based on a 250 µl volume. Bacteria to be tested were harvested from solid GC agar after 20-22 hrs growth, suspended in MEM, and  $1.5\text{--}2.5 \times 10^3$  CFU in 50 µl were added to each well. Plates were incubated at 37°C in 7% CO<sub>2</sub> for 1 hr, after which 50 µl of GCB were added and mixed. Two 50 µl aliquots were cultured on GC agar, and the average number of CFU recovered was determined. The bactericidal<sub>50</sub> titer was the concentration of antibody that resulted in a 50% reduction in the number of CFU recovered from wells that contained serum but no added test antibodies. Polyclonal rabbit antiserum against whole gonococci was used as a positive control, and antibodies that did not bind the target strain were used as negative controls. Heat-inactivated (HI) NHS and BBS were prepared by incubation at 56°C for 30 min, and were tested in parallel for each assay; none of the test or control antibodies had activity when HI serum was used.

**Agglutination assay** Agglutination titers were determined by the method of Pal *et al.* (134). Bacteria from primary subcultures of frozen stocks of Opa variants were suspended in PBS and passed through a 1.2 µm pore to remove aggregates. Filter suspensions were adjusted to an A<sub>600</sub> of 0.4. Test antibodies or antisera were serially diluted 2-fold and 10 µL of each dilution were incubated with 10 µL of bacteria ( $\sim 5 \times 10^6$  CFU) at 37°C in a microtiter plate for 45 min. Bacteria were incubated with the same dilutions of normal mouse sera (NMS) or affinity-purified polyclonal rabbit antibodies against heterologous HV<sub>2</sub> loops in parallel. After incubation, 5 µL were spotted on a glass slide and stained with HEMA3 (Protocol). The average number of aggregates per at

least three 40X fields was determined under light microscopy. Agglutination titers were defined as the highest dilution of test antibodies that caused greater than three times the number of aggregates seen in the same dilution of NMS or control antibodies. Comparisons between piliated and non-piliated variants of the same Opa type were also performed and identical results were obtained (data not shown).

***Passive protection experiments*** Female BALB/c mice 6-8 weeks of age (National Cancer Institute, Frederick, MD) were treated with 1.5 mg water-soluble 17 $\beta$ -estradiol (Sigma) and antibiotics to promote susceptibility to *N. gonorrhoeae* as described (172). In pilot experiments, 5-8 mice per group were inoculated vaginally with 10<sup>3</sup>, 10<sup>4</sup>, or 10<sup>5</sup> CFU of predominantly OpaB-variants that were pre-incubated in PBS with 250 or 500  $\mu$ g/ml of Ab<sub>HV2BD linear</sub> or in PBS alone for 20 min at 37°C. In subsequent experiments with loop-specific affinity-purified rabbit antibodies, bacteria (~ 5 x 10<sup>4</sup> CFU / ml) were preincubated in PBS with 250  $\mu$ g/ml of Ab<sub>HV2A linear</sub>, Ab<sub>HV2BD linear</sub>, or Ab<sub>SV linear</sub>. Twenty microliters of the suspension (~10<sup>3</sup> CFU and 5  $\mu$ g of antibodies) were then inoculated vaginally into mice (n = 7-15 mice per group). In studies with mouse antisera against cyclic peptides, mice were inoculated with a 20  $\mu$ l suspension containing 6 x 10<sup>3</sup> CFU that were preincubated with Ab<sub>HV2BD cyclic</sub>, Ab<sub>SV cyclic</sub>, or NMS (final dilution of antiserum or NMS, 1:30) (n = 10 - 11 mice per group). For all experiments, vaginal mucus was quantitatively cultured for *N. gonorrhoeae* daily for 3 days as described (91).

***Measurement of antibody duration*** In separate experiments, the amount of topically applied antibody recovered from the vagina was measured over time by inoculating 23 untreated, 6 week-old female BALB/c mice vaginally with 20  $\mu$ L of PBS containing 10  $\mu$ g of affinity-purified rabbit polyclonal Opa-specific antibodies. The

vaginas of 2-3 mice per time point were washed 3 times with 40  $\mu$ L PBS and the three samples from each mouse were pooled (~120  $\mu$ L) and centrifuged at 13,000 rpm for 3 min. Supernatants were frozen at -20°C. Control samples were collected from 3 untreated mice. The concentration of rabbit IgG in murine vaginal washes was measured with the Rabbit IgG Quantitative Kit ELISA (Bethyl Laboratories). Animal experiments were conducted in the laboratory animal facility at USUHS, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the USUHS Institutional Animal Care and Use Committee.

***Adherence assay*** ME180 cervical epithelial cells (ATCC, Manassas, VA) were grown to near confluency in 24-well tissue culture plates in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Quality Biological Inc., Gaithersburg, MD) and 2.2 g/L sodium bicarbonate. Non-piliated OpaB-expressing bacteria were subcultured from the freezer and passed once to GC agar before being suspended in McCoy's 5A medium supplemented with 2.2 g/L sodium bicarbonate and 5 mg/L  $\text{Fe}(\text{NO}_3)_3$  to an  $A_{600}$  of 0.07. Bacterial suspensions were diluted 1:10 and pre-incubated for 5 min with mouse antisera against HV<sub>2</sub> or SV cyclic peptides (test) or NMS (negative control) (final dilutions 1:30 and 1:100), or with 0.25  $\mu$ g/mL or 2.5  $\mu$ g/mL of Ab<sub>HV2BD linear</sub> (test) or Ab<sub>HV2C linear</sub> (negative control). Bacterial suspensions (500  $\mu$ l) were applied to cells (multiplicity of infection, 10:1). After 2 hrs at 37°C in 7% CO<sub>2</sub>, monolayers were washed four times with PBS to remove nonadherent bacteria, and cells were lysed with 0.5% saponin (Sigma). The number of cell-associated bacteria was determined by serial dilution, and culture of the saponin-treated suspensions. All assays

were performed in triplicate and the percent of cell-associated bacteria from the inoculum was compared between test and control wells.

***Statistical analysis*** A Fishers Exact test was used to compare the number of mice colonized in each experimental group in passive protection experiments. Differences in the number of gonococci recovered from mice and the recovery of cell-associated gonococci in tissue culture experiments were analyzed by the Student's t-test.

## ***Results***

### ***Antibodies against the SV loop and 4L loop are broadly reactive***

The 4L loop sequence of strain FA1090 is highly conserved with only a single amino acid difference between the 8 Opa proteins expressed by this strain. The SV loop is relatively well conserved with the OpaA and OpaK proteins sharing the same SV loop sequence and only 2-12 amino acid variations among the other 6 proteins (Figure 6). We hypothesized that the 4L and SV loops would therefore be useful for generating broadly reactive antibodies. To test this hypothesis we obtained affinity-purified rabbit polyclonal antibodies against linear peptides that correspond to the OpaAK SV (Ab<sub>SV linear</sub>) and 4L (Ab<sub>4L linear</sub>) loop sequences and assessed their specificity by western blot. As reported previously (165), antibodies against the fourth loop strongly recognized all of the Opa proteins of strain FA1090 except OpaE (Figure 7C). Ab<sub>SV linear</sub> bound strongly to denatured OpaA and OpaK, and also to OpaF and OpaI, which have an SV loop that is predicted to differ from the OpaAK SV sequence by 2 and 7 amino acids, respectively.

**Figure 6. Conservation of SV and HV<sub>2</sub> loop sequences.** The predicted amino acid sequences of the SV and HV<sub>2</sub> loops of the 8 Opa proteins of strain FA1090 are shown. The sequences of the 36-mer cyclic peptides used to generate SV- and HV<sub>2</sub><sub>BD</sub>-specific antisera are highlighted in grey. The sequences of the linear peptides used to generate affinity-purified rabbit antibodies against the SV and HV<sub>2</sub> loops are shown in light grey font.

Figure 6. Conservation of SV and HV<sub>2</sub> loop sequences.**SV Loop Sequences**

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OpaA    **a a e r i - t h d y p e p t g a k k g k - i s t v s d y f r n i r t h s i h**

OpaK    a a e r i - t h d y p e p t g a k k g k - i s t v s d y f r n i r t h s i h

OpaF    a a e r i - t h d y p e p t g a k k d k k i s t v s d y f r n i r t h s v h

OpaD    a a e r i - t h d y p e p t a p g k n k - i s t v s d y f r n i r t h s i h

OpaB    a a e r i - h t d y p e p t a p g k n k - i s t v s d y f r n i r t h s i h

OpaI    a y e h i - t r d y p d a a g a n k g k - i s t v s d y f r n i r t h s i h

OpaC    a y e h i r t r d y p d a a g a n q g k k i s t v s d y f k n i r t h s i h

OpaE    a y e h i - t r d y p d a a g a n q g k k i s t v s d y f k n i r t r s v h

**HV<sub>2</sub> Loop Sequences**

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OpaB    - **h s i d s t k k i t g t l t a y p s d a d a a v t v y p d - - g h p q k - n t y**

OpaD    - h s i d s t k k i t g t l t a y p s d a d a a v t v y p d - - g h p q k - n t y

OpaC    - h s i d s t k k t t e f l t a a g q d g - g a p t v y n - - - n g s t q - d a h

OpaF    r h s i d s t k k t t d v i t a p p t t s d g a p t t y n a - - n p q t q - n p y

OpaA    - h q v r s v e q e t t t v t t y l q s g k p s p i v - r g - - s t l k l - p h -

OpaE    - h q v h s m e k e t t t v t t y p s d g s a k t s v - p s - - e m p p k - p a y

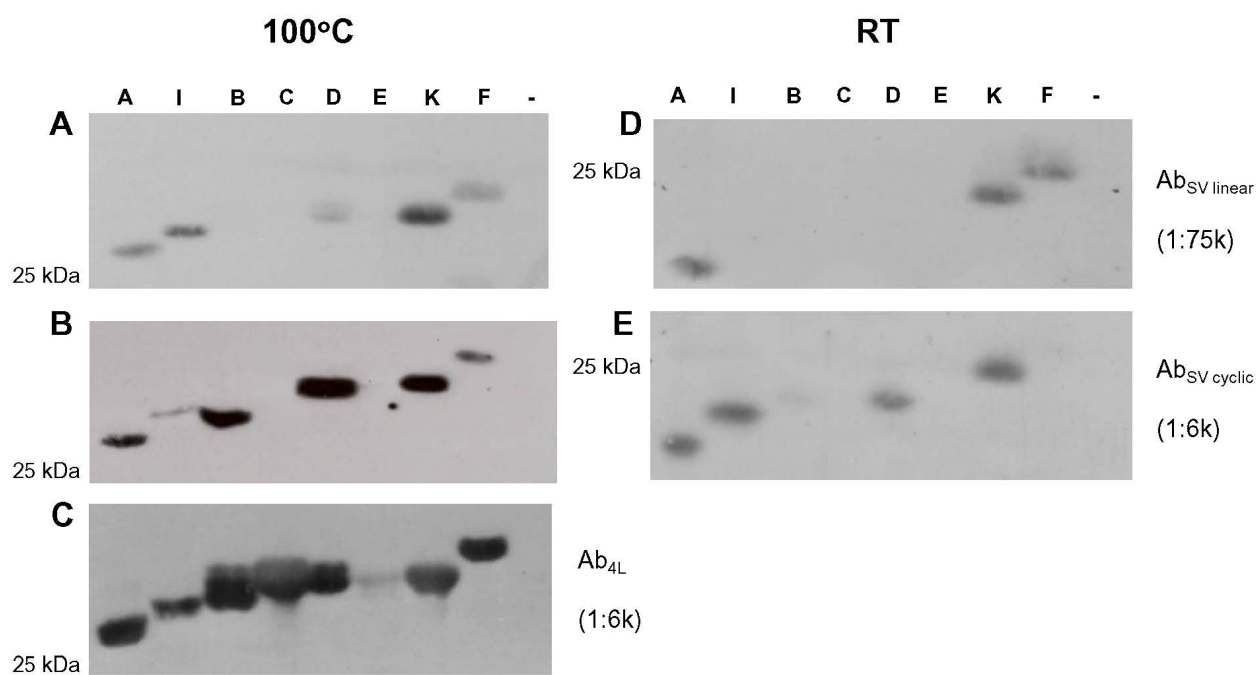
OpaK    - h q v h s m e k e t t t v t t y p s d g s a k t s v - p s - - e m p p k - p a y

OpaI    - h g i d s t k k t k d t l t a y h s a g - t k p t y y d d i d s g k t k k n t y



**Figure 7. Specificity of antibodies against the conserved Opa loops.** Cell lysates of an Opa-negative variant and each of the 8 Opa-positive variants of strain FA1090 were treated for 10 min at RT to preserve native conformations or 100°C to denature the proteins before being fractionated on an 11.5% SDS-PAGE gel. **(A and D)** Ab<sub>SV linear</sub> (1:75k), **(B and E)** Ab<sub>SV cyclic</sub> (1:6k), and **(C)** Ab<sub>4L linear</sub> (1:6k), were tested for recognition of the proteins by western blot. The broader reactivity of Ab<sub>SV cyclic</sub> compared to Ab<sub>SV linear</sub> is clearly shown whether native or denature Opa proteins are analyzed; Ab<sub>4L linear</sub> is even more broadly reactive in that it recognizes all denatured Opa proteins well except OpaE. None of the antibodies recognize a protein in the Opa-negative lane (-). The location of a 25 kDa molecular weight marker is indicated and denatured Opa proteins migrate at a slightly higher molecular weight consistent with well characterized heat modifiable nature of these proteins. The Ab<sub>4L linear</sub> immunoblot was kindly provided by Dr. Amy Simms.

Figure 7. Specificity of antibodies against the conserved Opa loops.



Ab<sub>SV linear</sub> weakly recognized OpaD, the predicted sequence of which differs from that of the OpaAK SV loop by 4 amino acids. When room temperature-treated samples were analyzed for reactivity, Ab<sub>SV linear</sub> recognized only OpaA, OpaK and OpaF. OpaD and OpaI proteins were not recognized. Ab<sub>SV linear</sub> did not recognize OpaB, which differs by 6 amino acids from the target peptide or OpaC or OpaE under either condition, which differ from the OpaAK SV sequence by 11-12 amino acids (Figures 7A and D).

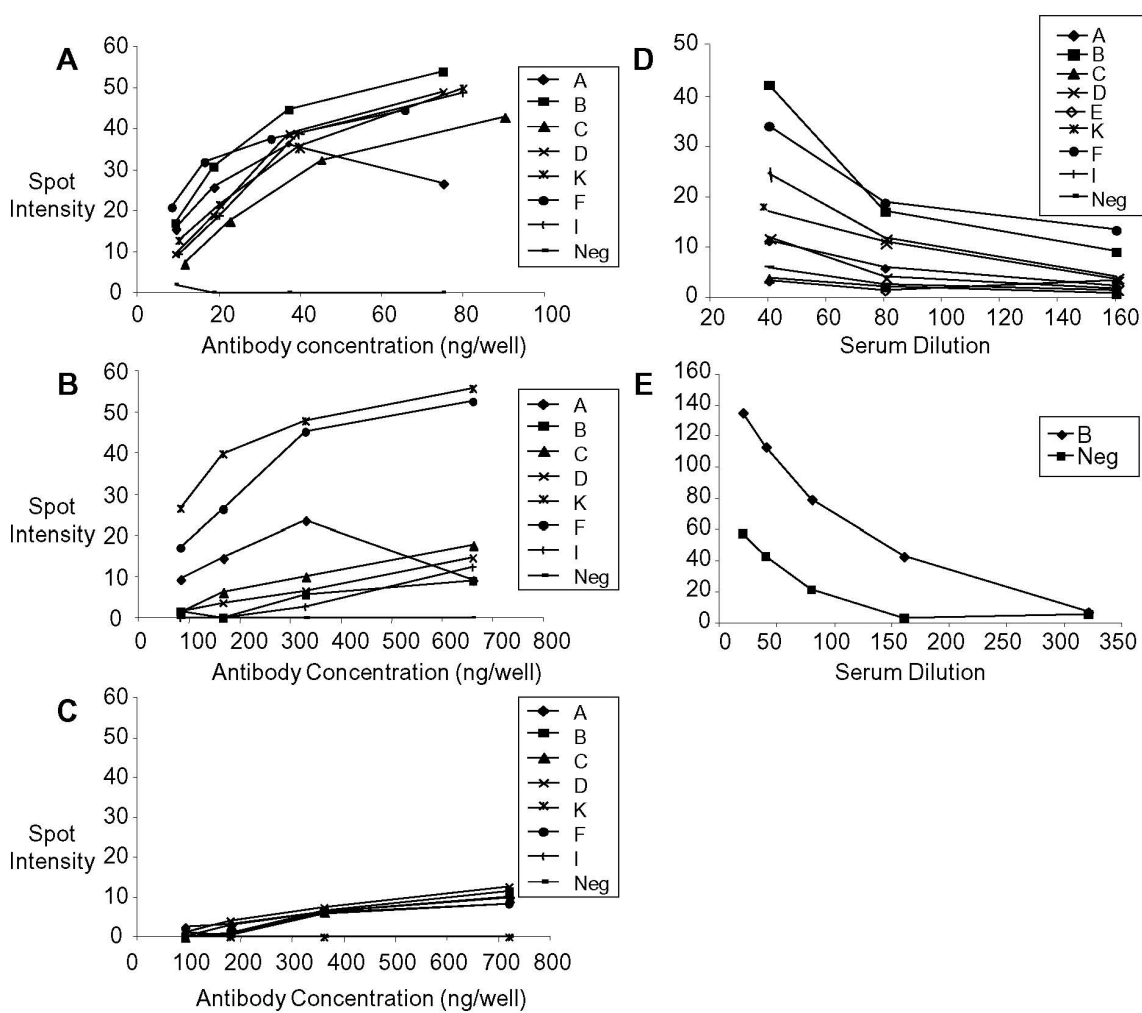
We also immunized mice with cyclic peptides that correspond to a longer surface-exposed region of the OpaAK SV loop that does not include any of the predicted transmembrane regions (Figure 6) to increase the likelihood of generating conformation-dependent antibodies that might be bactericidal (32). The resultant antiserum, Ab<sub>SV cyclic</sub>, was more broadly reactive than Ab<sub>SV linear</sub> and recognized all but 2 Opa proteins of strain FA1090 when denatured samples were analyzed (Figure 7B). Ab<sub>SV cyclic</sub> recognized Opa proteins with up to 7 amino acid differences compared to the cyclic SV peptide, but not OpaC or OpaE, which are the most divergent from OpaAK in the SV region (Figure 6). Ab<sub>SV cyclic</sub> recognized OpaA, OpaK, OpaD and OpaI proteins when RT-treated samples were used.

### ***SV-specific but not 4L-specific antibodies bind the bacterial surface***

The semi-quantitative SBI assay, which utilizes whole gonococci, was used to compare the concentration of antibodies needed to confer detectable binding to the different Opa variants of strain FA1090. Affinity-purified rabbit antibodies against linear HV<sub>2</sub> loop peptides (Ab<sub>HV2 linear</sub>) bound only the homologous Opa variants (Figure 8A) and not heterologous or Opa-negative variants (data not shown) in a dose-dependent manner as predicted. Ab<sub>SV linear</sub> bound only OpaA, OpaK and OpaF variants (Figure 8B); this

**Figure 8. Detection of antibody surface-binding by the SBI assay.** Opa-specific antibodies or antisera were serially diluted and incubated with defined Opa variants spotted onto nitrocellulose filters. Detection of antibody binding was with secondary antibodies conjugated to HRP followed by exposure to chemiluminescent substrate. Spot intensities were normalized as described in the Materials and Methods and plotted against the dilution of affinity-purified rabbit concentration of antibodies ( $\text{Ab}_{\text{HV2 linear}}$ ,  $\text{Ab}_{\text{SV linear}}$ , and  $\text{Ab}_{\text{4L linear}}$ ) or dilution of antisera ( $\text{Ab}_{\text{SV cyclic}}$  and  $\text{Ab}_{\text{HV2BD cyclic}}$ ): **(A)**  $\text{Ab}_{\text{HV2 linear}}$ , **(B)**  $\text{Ab}_{\text{SV linear}}$ , **(C)**  $\text{Ab}_{\text{4L linear}}$  **(D)**  $\text{Ab}_{\text{SV cyclic}}$ , **(E)**  $\text{Ab}_{\text{HV2BD cyclic}}$ . The dose-dependent binding of each of the  $\text{HV}_2$ -specific antibodies against the homologous variant is shown in panel A. These antibodies did not bind heterologous variants (data not shown) or an Opa-negative variant, for which the reaction of  $\text{Ab}_{\text{HV2BD linear}}$  against Opa-negative variants is shown as a representative example.  $\text{Ab}_{\text{SV linear}}$  bound OpaA, OpaF, and OpaK variants well, but the concentration of antibodies needed to detect binding was higher than that needed for antibodies against the  $\text{HV}_2$  loops. In panel C, antibodies to the 4L linear peptide showed little or no detectable binding. A larger subset of Opa variants was recognized by antiserum against the cyclic SV peptide than antibodies induced by a linear peptide (compare panels B and D). Dose dependent binding to the constitutively OpaB-expressing strain was observed with  $\text{Ab}_{\text{HV2BD cyclic}}$ , with maximal binding at a  $< 1:100$  dilution of antisera as shown in panel E.  $\text{Ab}_{\text{HV2BD cyclic}}$  did not bind the Opa-deficient strain. All results shown are representative of at least two independent experiments.

Figure 8. Detection of antibody surface-binding by the SBI assay.



result is consistent with western blot results against unheated lysates in which native conformations are preserved. Approximately 10-fold higher concentrations of Ab<sub>SV linear</sub> were required to detect antibody binding to whole gonococci compared to Ab<sub>HV2 linear</sub> (compare Figures 8A and 8B). No specific binding was seen with Ab<sub>4L linear</sub> at any concentration analyzed (Figure 8C).

The reactivity of the mouse antiserum against the cyclic SV loop peptide in the SBI assay mirrored the broader reactivity observed with this antiserum on western blots. Ab<sub>SV cyclic</sub> bound to intact OpaA, OpaB, OpaD, OpaF, OpaK and OpaI variants above background in the SBI assay at a dilution of 1:40, while OpaC, OpaE, and Opa-negative variants were not recognized by Ab<sub>SV cyclic</sub> (Figure 8D). We also tested mouse antisera against a cyclic peptide that corresponds to the HV<sub>2</sub> loop of OpaB/D. As predicted, Ab<sub>HV2BD cyclic</sub> bound to OpaB variants better than Opa-negative variants (Figure 8E). Consistent with the increased surface exposure of the HV<sub>2</sub> loop, the dilution of Ab<sub>HV2BD cyclic</sub> required to produce a spot of similar intensity when tested against OpaB variants was 5-fold higher than that of the Ab<sub>SV cyclic</sub> antiserum (compare Figures 8D and 8E).

IFA staining was performed as a second measure of surface-binding. Consistent with the results obtained by SBI, Ab<sub>SV linear</sub> (2.2 µg/mL) bound OpaA variants as well as OpaK and OpaF-expressing gonococci but none of five other Opa variants of strain FA1090. Ab<sub>SV cyclic</sub> bound OpaA, OpaB, OpaD, OpaF, OpaK and OpaI variants at a 1:30 dilution but not OpaC or Opa-negative variants (Table 1). As with rabbit antibodies against linear peptides, mouse antiserum against the cyclic HV<sub>2BD</sub> peptide bound OpaB variants at a higher dilution (1:100) than did mouse antiserum against the cyclic SV loop peptide (1:30), a result that reflects the better surface exposure of the HV<sub>2</sub> loop. Ab<sub>4L linear</sub>

Table 1. Surface-binding of SV loop-specific antibodies as assessed by SBI and IFA

	<b>Ab<sub>SV</sub> linear<sup>a</sup></b>		<b>Ab<sub>SV</sub> cyclic<sup>b</sup></b>	
	<b>IFA</b>	<b>SBI</b>	<b>IFA</b>	<b>SBI</b>
<b>OpaA</b>	+	+	+	+
<b>OpaB</b>	-	-	+	+
<b>OpaC</b>	-	-	+/-	-
<b>OpaD</b>	-	-	+	+
<b>OpaE</b>	nt <sup>c</sup>	nt <sup>c</sup>	nt <sup>c</sup>	-
<b>OpaF</b>	+	+	+	+
<b>OpaK</b>	+	+	+	+
<b>OpaI</b>	-	-	+	+

<sup>a</sup>affinity-purified polyclonal rabbit antibodies<sup>b</sup>mouse antisera<sup>c</sup>not tested

did not bind any Opa-expressing gonococci at concentrations of 1.2 µg/mL or 2.4 µg/mL (data not shown), a result that confirms the negative SBI results with these antibodies. Antibodies specific for the HV<sub>2</sub> loop of each Opa variant were used as positive controls (0.87 - 1.2 µg/mL) in all IFA assays and to confirm that the Opa protein being tested was in fact expressed by the bacteria on the slides when results with Ab<sub>SV linear</sub>, Ab<sub>SV cyclic</sub> and Ab<sub>4L linear</sub> were negative.

In summary, the IFA and SBI results were undistinguishable and confirmed the surface-exposure of the SV and HV<sub>2</sub> loops. The need for increased concentration of SV loop-specific antibodies to detect surface binding suggests that the SV loop is less accessible than the HV<sub>2</sub> loop. We also conclude that the fourth loop may not be accessible to antibody despite its predicted surface-exposure. Alternatively, the linear 4L peptide may not induce antibodies that recognize conformational epitopes present in the native protein. Finally, we also demonstrated that the cyclic SV peptide induced more broadly reactive antibodies compared to a linear peptide.

### ***Bactericidal and agglutination activities***

We next investigated whether cyclic or linear peptide-derived antibodies against the SV loop or the HV<sub>2</sub>-loops would be bactericidal. Disappointingly, Ab<sub>SV linear</sub> were not bactericidal against any of the variants tested at concentrations ranging from 4.5 - 120 µg/mL. Surprisingly, the use of cyclic peptides to induce bactericidal antibodies against the SV or HV<sub>2BD</sub> loops was also not successful with no bactericidal activity detected at dilutions from 1:9 – 1:576. Analysis of mouse antisera to cyclic loop peptides showed IgG2a>IgG1>IgG2b>IgG3.

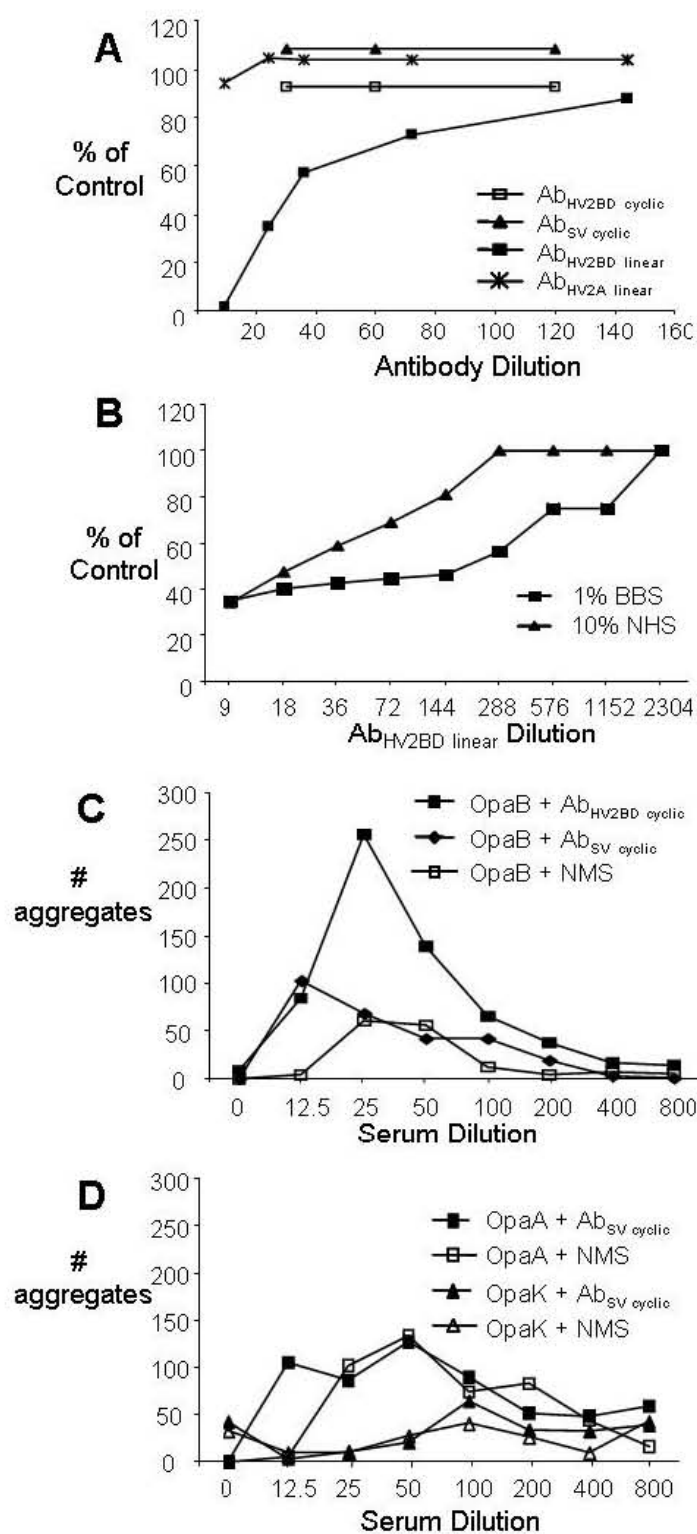


In contrast to mouse antisera against cyclic peptides, rabbit antibodies against a linear peptide that corresponds to the HV<sub>2</sub> loop of OpaB, Ab<sub>HV2BD linear</sub>, were bactericidal against OpaB variants with a bactericidal<sub>50</sub> concentration of 40.4 µg/mL. Antibodies against the HV<sub>2</sub> loop of Opa I, Ab<sub>HV2I linear</sub>, also demonstrated bactericidal activity against a homologous variant with a bactericidal<sub>50</sub> concentration of 26 µg/mL. No other HV<sub>2</sub> linear-specific antibodies were bactericidal against the corresponding homologous Opa variant. Rabbit polyclonal antisera against whole FA1090 bacteria showed high levels of bactericidal activity against all Opa variants tested (positive control).

Strain FA1090 is inherently resistant to the bactericidal activity of NHS due to the binding of the complement regulatory protein human C4b-binding protein (C4BP) to its porin (144). This interaction is host-restricted (132). Therefore, to better mimic events that might occur when testing the activity of these antibodies in the mouse infection model, we utilized strain FA1090<sub>F62por5-8</sub>, which produces a recombinant hybrid porin that does not bind hC4BP and is thus serum sensitive. As observed with wild type FA1090 bacteria, Ab<sub>HV2BD linear</sub> but not the mouse antiserum Ab<sub>HV2BD cyclic</sub> or Ab<sub>SV cyclic</sub> was bactericidal against strain FA1090<sub>F62por5-8</sub> in the presence of NHS. Ab<sub>HV2A linear</sub>, which, like Ab<sub>HV2BD linear</sub>, are affinity purified rabbit polyclonal antibodies, was also not bactericidal for this serum sensitive derivative of strain FA1090 as predicted (Figure 9A). To further investigate this host restriction, we also compared NHS with BBS as a nonhuman complement source. For these experiments, we utilized a constitutive OpaB-expressing derivative of wild type strain FA1090 and a strain in which all the *opa* genes have been inactivated. Consistent with the host restriction for hC4BP, Ab<sub>HV2BD linear</sub> exhibited an 8-fold increase in activity when BBS was used. The bactericidal<sub>50</sub> titers

**Figure 9. Bactericidal and agglutination activities.** The bactericidal activity of loop-specific antibodies was measured against a serum sensitive FA1090 strain and wild-type FA1090 bacteria and with human and non-human sources of complement. **(A)** FA1090<sub>F62por 5-8</sub> OpaB variants incubated with loop-specific antibodies in 1% NHS. Antisera against the SV or HV<sub>2BD</sub> cyclic loops were not bactericidal. Ab<sub>HV2BD linear</sub>, in contrast, had a bactericidal<sub>50</sub> titer of 1:36 which equals 28.1 µg/mL. Ab<sub>HV2A linear</sub>, which was used as a heterologous control, did not kill FA1090<sub>F62por 5-8</sub> OpaB variants. **(B)** The constitutively OpaB-expressing strain of FA1090 (SR) incubated with Ab<sub>HV2BD linear</sub> in the presence of NHS or BBS. Use of a non-human complement source resulted in greater bactericidal activity for Ab<sub>HV2BD linear</sub> compared to human complement, with an ~8-fold increase in bactericidal<sub>50</sub> titers (1:36 or 28.1 µg/mL for NHS and 1:288 or 3.5 µg/mL for BBS). Ab<sub>HV2BD linear</sub> showed no bactericidal activity when HI-NHS and BBS were used. Results shown are representative of at least two independent experiments and are expressed as the percent of bacteria recovered compared to wells with no antibody present. **(C)** Agglutination activity was measured by incubating bacteria with increasing dilutions of loop-specific antibodies and enumerating the number of bacterial aggregates in stained aliquots from at least three 40X fields. The average number of bacterial aggregates is shown. OpaB variants with increasing dilutions of Ab<sub>HVBD cyclic</sub>, Ab<sub>SV cyclic</sub>, or NMS. The agglutination titer for Ab<sub>HV2BD linear</sub> is ~1:200 and is 1:12.5 for Ab<sub>SV cyclic</sub>. **(D)** Incubation of OpaA or OpaK variants with Ab<sub>SV cyclic</sub> or NMS. The agglutination titer for Ab<sub>SV cyclic</sub> against OpaA variants was 1:12.5. Although Ab<sub>SV cyclic</sub> bound OpaK, no agglutination activity was observed for OpaK variants when compared to the NMS control.

Figure 9. Bactericidal and agglutination activities.



against the OpaB-expressing strain were 1:36 (28 µg/mL) and 1:288 (3.5 µg/mL) when NHS and BBS, respectively, were compared in the same assay (Figure 9B). No bactericidal activity was elicited by Ab<sub>HV2BD linear</sub> against the Opa-deficient strain with either NHS or BBS (data not shown).

The capacity to agglutinate bacteria may facilitate shedding of bacteria in vaginal secretions and thus may be another important effector function of antibodies. Ab<sub>HV2BD cyclic</sub> agglutinated OpaB variants at a titer of 1:200 with ~40 aggregates per 40X field compared to only ~4 aggregates per field with the same dilution of NMS. (Figure 9C). In contrast to the antisera against cyclic peptides, Ab<sub>HV2BD linear</sub> did not agglutinate OpaB variants at dilutions as low as 1:2 (500 µg/mL) (data not shown). Ab<sub>SV cyclic</sub> agglutinated OpaA and OpaB variants at a titer of 1:12.5 when compared to NMS, but not OpaK variants (Figures 9C and D). Ab<sub>SV linear</sub> did not agglutinate any Opa variants tested (data not shown).

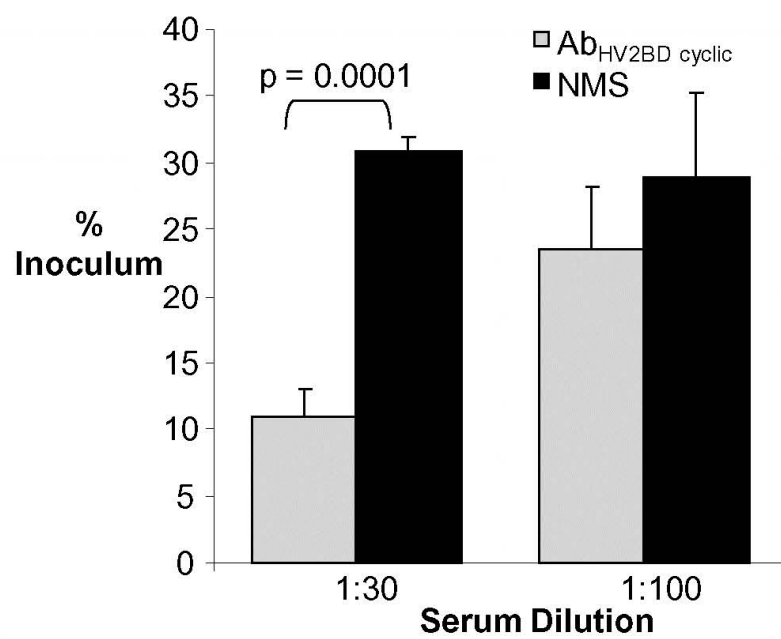
***HV<sub>2</sub>-specific but not SV-specific antibodies reduce adherence to cultured cervical cells***

The capacity of Opa-specific antibodies to inhibit Opa-mediated adherence and invasion may also be protective. Most Opa proteins mediate invasion of human tissue culture cells via binding to human carcinoembryonic cellular adhesion molecules (CEACAMs) (46). To test whether Opa loop-specific antibodies can block gonococcal interactions with endocervical cells, the constitutive OpaB-expressing strain was incubated with Ab<sub>HV2BD linear</sub>, Ab<sub>HV2BD cyclic</sub>, or Ab<sub>SV cyclic</sub> and inoculated onto ME180 cells. Preincubation with a 1:30 dilution of Ab<sub>HV2BD cyclic</sub>, but not Ab<sub>SV cyclic</sub> significantly decreased the number of cell-associated bacteria as compared to NMS (Figure 10).

**Figure 10. Antibody-mediated inhibition of gonococcal association with tissue**

**culture cells.** OpaB variants were incubated with Ab<sub>HV2BD cyclic</sub> or NMS for 5 min before addition to confluent ME180 cervical epithelial cells at an MOI of 10. Incubation with antiserum against the HV<sub>2BD cyclic</sub> peptide decreased the total number of cell-associated bacteria when an antiserum dilution of 1:30, but not 1:100 was used. Results are expressed as the percent adherence relative to the inoculum. Student's t-test was used to assess statistical differences.

Figure 10. Antibody-mediated inhibition of gonococcal association with tissue culture cells.



Inhibition was not observed when a higher dilution of Ab<sub>HV2BD cyclic</sub> was tested, and was thus dose-dependent. In contrast there was no decrease in the number of cell-associated bacteria when bacteria were treated with 0.25 µg/mL or 2.5 µg/mL of Ab<sub>HV2BD linear</sub> compared to the Ab<sub>HV2C linear</sub>, which does not bind OpaB variants (data not shown). We conclude Ab<sub>HV2BD cyclic</sub>, but not Ab<sub>HV2BD linear</sub> block OpaB-mediated interactions with human endocervical cells.

### *Passive protection studies*

The CEACAM residues that are important for Opa-mediated adherence are not conserved in the murine CEACAM1 (165, 185), and consistent with this host restriction, we have not observed Opa-mediated adherence to two different murine epithelial cell lines (see Chapter 3, Fig. 16). Female mice can be experimentally infected with *N. gonorrhoeae* despite the absence of human CEACAMs (91, 165), and Opa-specific antibodies could prevent or reduce colonization of mice through bactericidal activity, opsonization for increased phagocytic uptake, or agglutination with subsequent shedding. Additionally, because selection for Opa expression occurs during experimental infection of BALB/c mice (165), Opa-specific antibodies may block other functions that have yet to be identified (172). We therefore tested the capacity of Opa loop-specific antibodies to reduce gonococcal colonization of BALB/c mice when administered vaginally as done for passive protection experiments against other sexually transmitted pathogens (134, 136, 184, 193).

First, to determine how long antibodies remain in the vagina after topical administration, groups of mice were inoculated vaginally with 20 µl of PBS containing 10 µg of Ab<sub>HV2 linear</sub>, and the amount of rabbit IgG in vaginal washes between 2 min and

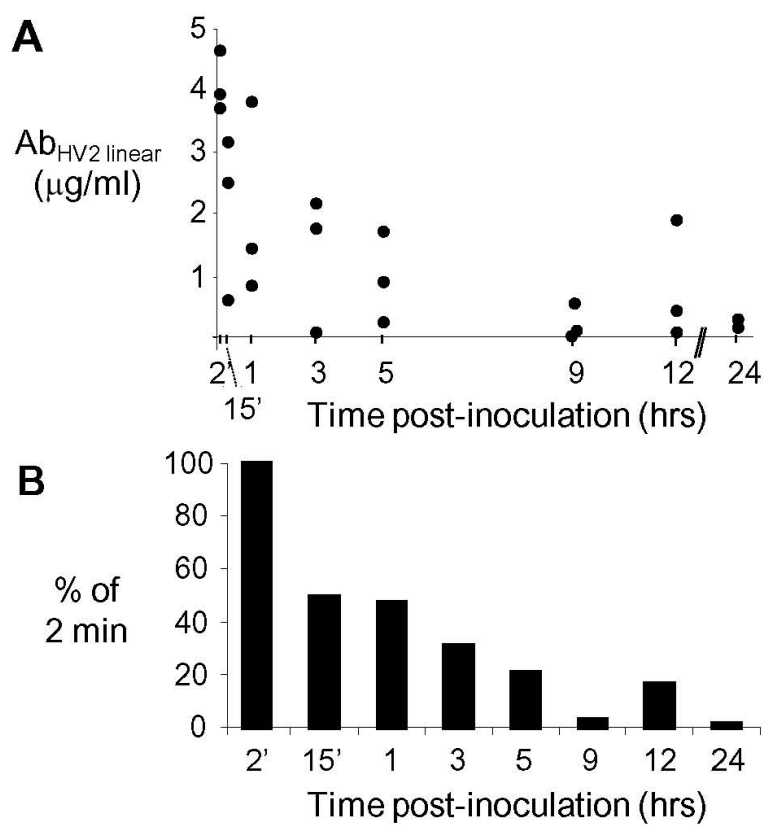
24 hrs post administration was measured. A 50% decrease in the amount of rabbit IgG recovered was observed between 2 and 15 min post-inoculation. Antibody levels were maintained for at least another 45 min, after which a gradual decline was observed over the next 4 hours. Low levels of antibody were detected in all but one mouse at 9, 12, and 24 hrs post-inoculation (Figure 11). No rabbit IgG was detected in vaginal washes from untreated control mice. There was no correlation between persistence of antibody and the stage of estrous of the test mice at the time of inoculation. Based on these data we concluded that antibody must function within the first 24 hrs to be effective, and thus chose to analyze colonization loads only at early time points (days 1, 2, and 3 post-inoculation). We next performed pilot experiments to optimize the bacterial dose. Mice were inoculated with  $10^3$ ,  $10^4$ , or  $10^5$  OpaB variants of strain FA1090. Eighty five percent of mice inoculated with  $10^3$  CFU maintained colonization for three days. We therefore chose this bacterial dose for subsequent experiments.

We first tested Ab<sub>SV linear</sub> to address our main objective of developing broadly-reactive protective antibodies against *N. gonorrhoeae*. OpaA variants were used since Ab<sub>SV linear</sub> bound OpaA variants (Figure 8B and Table 1). Ab<sub>HV2A linear</sub>, and Ab<sub>HV2BD linear</sub> were tested for comparison and as a negative control, respectively. Mice were inoculated with  $10^3$  OpaA variants that were preincubated with Ab<sub>SV linear</sub>, Ab<sub>HV2A linear</sub>, or Ab<sub>HV2BD linear</sub>, which does not bind OpaA. There was no significant difference in the number of gonococci recovered on days 1 and 2 post-inoculation when results from the Ab<sub>SV linear</sub> and Ab<sub>HV2A linear</sub> groups were compared to the Ab<sub>HV2BD linear</sub> control group (Figure 12A). We also tested Ab<sub>SV cyclic</sub>, which has broader reactivity than Ab<sub>SV linear</sub>, possibly due to the



**Figure 11. Duration of topically applied antibodies in the vagina.** Mice were inoculated intravaginally with 10 µg of Ab<sub>HV2 linear</sub> and vaginal washes were collected at the indicated time points. Rabbit antibodies were detected in all mice for at least 5 hrs post-inoculation, with ~50% remaining 1 hr after administration. A few mice had detectable antibodies for as long as 24 hrs post-inoculation. The limit of detection was 7.8 ng/ml. **(A)** Concentration of rabbit IgG (µg/ml) in mouse vaginal washes was determined in duplicate by capture ELISA. **(B)** The percent of antibody remaining relative to the average 2 minute value is shown for each time point. Results are combined from two experiments and each animal was used for a single time point (n = 2-3 mice per time point).

Figure 11. Duration of topically applied antibodies in the vagina.

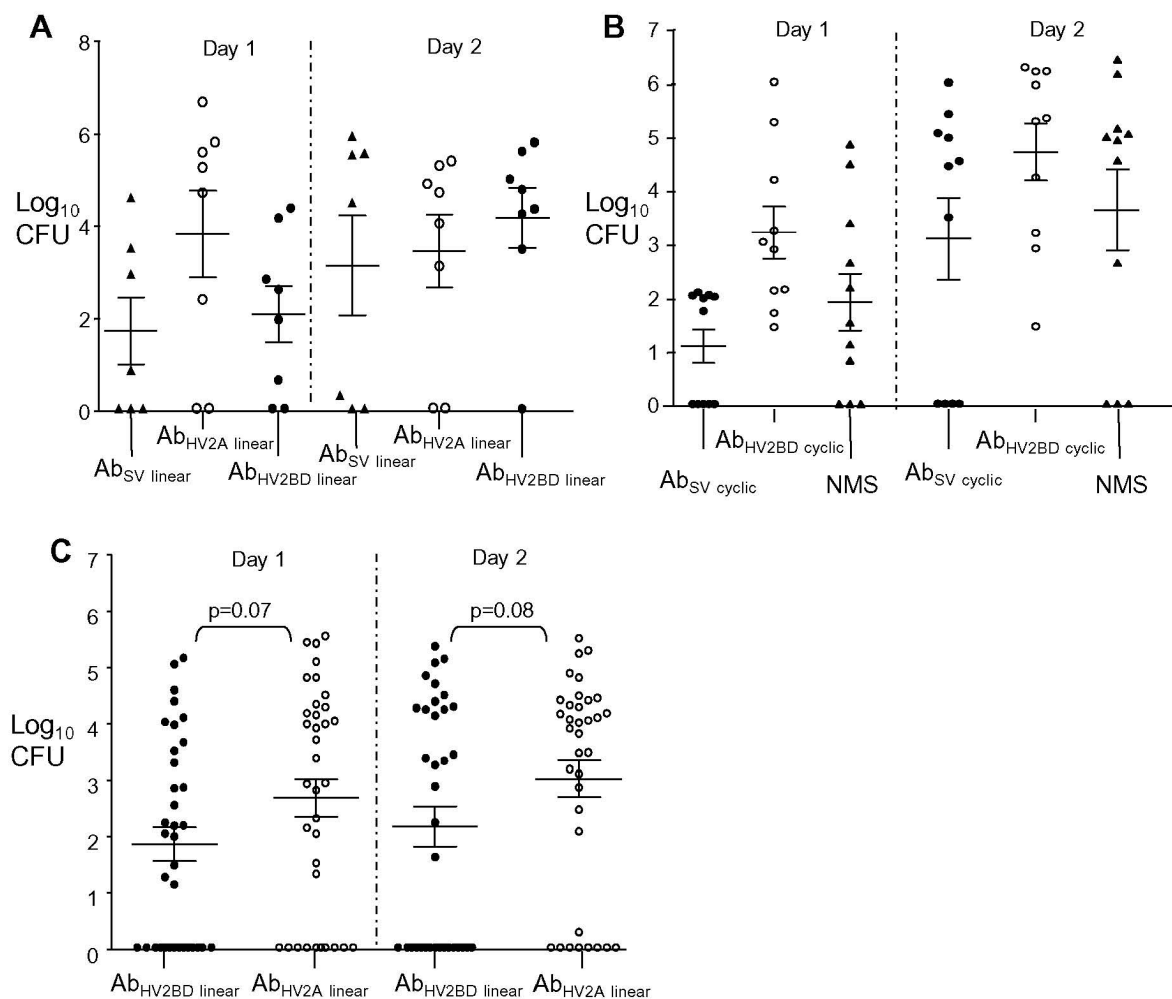


recognition of shared conformational epitopes among Opa proteins. We thus hypothesized Ab<sub>SV cyclic</sub> might be more effective than Ab<sub>SV linear</sub>. Ab<sub>SV cyclic</sub> bound OpaB variants well in the SBI and IFA assay, and we therefore assessed the capacity of Ab<sub>SV cyclic</sub> and Ab<sub>HV2BD cyclic</sub> to passively protect mice from OpaB variants compared to OpaB variants incubated in NMS. Incubation of OpaB variants with Ab<sub>HV2BD cyclic</sub> or Ab<sub>SV cyclic</sub> did not result in a significant difference in colonization load of test mice compared to the NMS control group (Figure 12B). There was no difference in the number of mice that were colonized on day 3 in either experiment with 57% and 64% colonization in groups treated with SV loop-specific antibodies compared to 88% and 82% colonization in control groups ( $p \geq 0.28$ ) (data not shown). In summary, these results demonstrate that despite binding to the bacterial surface, antibodies against the SV loop have limited capacity to reduce infection by *N. gonorrhoeae*, even when a cyclic peptide is used to better mimic the loop conformation.

Results from initial pilot studies suggested inoculation of mice with  $10^3$  CFU of OpaB variants incubated with 5  $\mu$ g Ab<sub>HV2BD linear</sub> would be protective in passive protection studies. Ab<sub>HV2BD linear</sub> were also bactericidal, unlike Ab<sub>HV2BD cyclic</sub>, and therefore, we performed a larger scale experiment, in which mice were inoculated with OpaB variants preincubated with Ab<sub>HV2BD linear</sub> or Ab<sub>HV2A linear</sub>, which do not bind OpaB variants. Significantly fewer bacteria were recovered from mice in the Ab<sub>HV2BD linear</sub> test group compared to mice for which Ab<sub>HV2A linear</sub> was used ( $p \leq 0.03$ ) on days 1 and 2 post-inoculation. However, in two subsequent experiments there was no significant difference

**Figure 12. Recovery of bacteria from mice challenged with gonococci incubated with Opa loop-specific antibodies.** Mice were inoculated with OpaA or OpaB variants that were preincubated with antibodies against linear or cyclic Opa SV or HV<sub>2</sub>-loop sequences. **(A)** OpaA variants with Ab<sub>SV linear</sub>, Ab<sub>HV2A linear</sub>, or Ab<sub>HV2BD linear</sub>. There was no difference in the number of gonococci recovered from mice in the Ab<sub>SV linear</sub> and Ab<sub>HV2A linear</sub> test groups compared to the Ab<sub>HV2B linear</sub> control group on days 1 or 2 post-inoculation ( $p \leq 0.14$ ). **(B)** OpaB variants with Ab<sub>HV2BD cyclic</sub>, Ab<sub>SV cyclic</sub> or NMS. There was no difference in the number of gonococci recovered from mice treated with Ab<sub>SV cyclic</sub> compared to the control (NMS) group ( $p \leq 0.20$ ) on days 1 or 2 post-inoculation. **(C)** OpaB variants with Ab<sub>HV2BD linear</sub> and Ab<sub>HV2A linear</sub>. Shown are combined data from three independent experiments that showed no difference in the number of bacteria recovered from each group on days 1 and 2 post-inoculation at the level of  $p = 0.07$  and  $p = 0.08$ , respectively ( $n = 7-15$  mice / group in each experiment; total number = 35 mice / group). Significant reduction in colonization occurred in only one of these experiments in which Ab<sub>HV2BD linear</sub> reduced the colonization load as compared to Ab<sub>HV2A linear</sub> at a level of  $p = 0.03$  and  $p = 0.02$  days 1 and 2, respectively. Symbols indicate a single animal and horizontal bars indicate the group mean with the SEM shown. Statistical differences were analyzed by a two-tailed Student's *t* test.

Figure 12. Recovery of bacteria from mice challenged with gonococci incubated with Opa loop-specific antibodies.



in colonization load recovered on days 1 or 2 (data not shown). Statistical analysis of combined data from all three experiments showed no significant decrease in recovery of gonococci from mice inoculated with Ab<sub>HV2BD linear</sub>-treated OpaB variants on days 1 and 2 post-inoculation with  $p = 0.07$  and  $p = 0.08$ , respectively, compared to mice inoculated with Ab<sub>HV2A linear</sub>-treated bacteria (Figure 12C). There was also no difference in the number of mice colonized in each group with 60% of the Ab<sub>HV2BD linear</sub> and 71% of the Ab<sub>HV2A linear</sub>-treated mice infected on day 1 ( $p = 0.45$ ). In summary, while initial experiments suggested antibodies against the HV<sub>2BD</sub> loop could decrease the colonization load *in vivo*, we were unable to consistently demonstrate protection with these antibodies. We also considered the possibility that subpopulations of gonococci that express a different Opa protein than that of target variant were responsible for the colonization of test groups. However, the distribution of Opa phenotypes of vaginal isolates on day 1 did not differ significantly from that of the inoculum in any experiment, from which we conclude that escape variants were not responsible for establishing infection.

### Discussion

*N. gonorrhoeae* is a highly successful Gram-negative bacterium that is noted for the antigenic variability of its surface and the frequency by which it causes repeat infections. Opa proteins are expressed during infection and have two conserved loops, the SV and 4L loops, which could be targeted in a vaccine. While the SV and 4L loops are smaller than the immunodominant HV loops, both are predicted to be surface-exposed (117). Here we analyzed antibodies against peptides that correspond to the SV and 4L

loops for the capacity to bind to gonococci that express different Opa proteins and for correlates of antibody-mediated protection. We also compared the activity of antibodies against cyclic versus linear peptides with the prediction being that cyclic peptides may better mimic loop conformation. Antibodies against linear or cyclic peptides that correspond to the SV loop recognized intact gonococci as assessed by two different methods, and promisingly, antibodies generated against cyclic SV peptides bound the surface of 6 of the 8 Opa variants of strain FA1090, including Opa variants with 7 amino acid differences from the target peptide. In contrast, 4L-specific antibodies did not bind the bacterial surface. Therefore, while predicted to be surface-exposed, the 4L may be poorly accessible or critical conformational epitopes were not reproduced in the linear 4L peptide.

The cyclic SV loop peptide also induced antibodies with agglutination ability, while the SV linear peptide did not. The broader reactivity and agglutinating ability of Ab<sub>SV cyclic</sub> may be explained by the fact that the cyclic SV peptide was longer (36 amino acids) than the linear peptides (20 amino acids) and included more conserved regions of the loop. Cyclic peptides may therefore carry more conserved epitopes, T-cell epitopes, and possibly conformational epitopes that are shared by Opa proteins with a less related primary sequence. Interestingly, bactericidal activity was only exhibited by antibodies against linear peptides. This result was in contrast to the demonstration that cyclic peptides were more successful for inducing bactericidal antibodies against the class 1 protein of *N. meningitidis* (32). With regard to the difference in bactericidal activity between Ab<sub>HV2BD linear</sub> and Ab<sub>HV2BD cyclic</sub>, one should consider the fact that the antibodies were produced in different animal species and that Ab<sub>HV2BD linear</sub> were high titer affinity-

purified antibodies. We also cannot rule out the possibility that the adjuvant used may influence whether bactericidal antibodies are produced. Antibodies generated by the linear or cyclic SV loop peptides were not bactericidal in the presence of either NHS or rabbit serum, the latter of which should by-pass the host-restricted serum resistance of this strain. The use of a different adjuvant might improve the potential of the SV loop as a vaccine target by promoting the induction of bactericidal antibodies.

We also showed that the species used as the complement source in bactericidal assays is important when examining the bactericidal activity of vaccine-induced antibodies against SR strains of *N. gonorrhoeae*. Some P1B strains, like strain FA1090 are resistant to NHS due to the binding of human C4BP to the P1B molecule (144), which reduces activation of the classical pathway and subsequent bacteriolysis (145). Here we demonstrated that Ab<sub>HV2BD linear</sub> killed OpaB variants of a SS derivative of strain FA1090 better than SR wild type OpaB variants when NHS was used as the complement source. Ab<sub>HV2BD linear</sub> was also more bactericidal against OpaB variants of strain FA1090 when rabbit serum was used. These results illustrate the importance of considering the complement source in bactericidal assays designed to predict vaccine efficacy in humans versus laboratory animals. Ideally, antibodies that are strongly bactericidal against SR strains in the presence of NHS are desired.

A vaccine-induced immune response against Opa proteins could also block Opa-mediated adherence and invasion, which has been the focus of vaccine studies on *N. meningitidis* Opa proteins. de Jonge *et al.* (43, 44) reported that antibodies raised against purified recombinant Opa proteins, outer membrane protein vesicles containing Opa proteins, or liposomes containing recombinant Opa proteins from *N. meningitidis* elicited



an antibody response, which while not always bactericidal, blocked Opa-CEACAM interactions on tissue culture cells (44). Here we showed that Ab<sub>HV2BD cyclic</sub> but not SV-specific antibodies decreased the total number of bacteria associated with CEACAM-expressing endocervical cells. The inability to block adherence with SV-specific antibodies is in accordance with studies on *N. meningitidis* Opa proteins that show the SV loop is not involved in CEACAM-binding (21). Other undefined Opa protein functions are likely to exist, however, that could perhaps be blocked by an SV loop-specific immune response. For example, Opa-expressing gonococci are preferentially recovered from the urethra of experimentally infected male volunteers (92, 176) and during lower genital tract infection of female mice (165). The basis for this selection is not clear, and the inability to detect CEACAMs on primary male urethral cells (75) and the dissimilarities between human and murine CEACAM1 (73, 185) suggest Opa proteins may play other important roles during infection such as evasion of host innate defenses (20).

Finally, we also tested the capacity of the Opa loop-specific antibodies to prevent or reduce colonization in the estradiol-treated mouse model of gonococcal infection, which should allow one to measure protection mediated by bactericidal activity, opsonophagocytosis, agglutination, or the blocking of unidentified advantages conferred by Opa expression *in vivo* (165). None of the antibodies tested significantly reduced the colonization load or number of mice colonized when mixed with the homologous variant prior to vaginal inoculation. Ab<sub>HV2BD linear</sub> were the only bactericidal antibodies that were tested *in vivo* and although promising results were initially obtained, these results were not reproducible. Following vaginal application, we demonstrated that persistence of

antibody was for < 24 hrs. This result is similar to that reported by Sherwood *et al.* (164), and illustrates the technical limitation of the approach we used in our passive protection experiments. For example, clearance via increased opsonophagocytosis might not occur in this time frame since phagocytes are not detected in infected murine tissue until 2 to 5 days post-inoculation (172). Protection may have been observed if the antibodies had persisted longer in the vagina. Systemic delivery of antibodies to the vagina has been successful for others. For example, monoclonal IgA against *Chlamydia trachomatis* was detected in mouse vaginal secretions for up to 48 hrs when delivered intraperitoneally (134), and Parr *et al.* (136) reported intraperitoneal administration of IgG resulted in the detection of specific IgG 48 hours later in vaginal secretions at levels equal to 3% of that found in the vaginal secretions of immunized mice. We have detected rabbit IgG in vaginal washes for as long as 60 hrs after intraperitoneal or intravenous injection of high titer rabbit polyclonal antiserum against whole gonococci (B.T. Mocca and A.E. Jerse, unpublished data). However, we did not detect gonococcal-specific antibodies in vaginal washes following intravenous injection of the affinity-purified HV<sub>2</sub>-specific rabbit antibodies used in this study, and we therefore chose to deliver the antibodies topically.

In summary, we have demonstrated that broadly-reactive antibodies can be generated against a relatively conserved Opa protein loop that bind to the bacterial surface and have agglutination ability. These antibodies could potentially recognize many Opa variants produced by different gonococcal strains and therefore, the use of other adjuvants or strategies to induce high titered SV loop-specific antibodies with bactericidal activity is

warranted. The *in vitro* and *in vivo* experiments described here should be useful in the development of other vaccine antigens against gonorrhea.

### *Acknowledgements*

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# **Chapter 3: Opacity Proteins Increase *Neisseria gonorrhoeae* Fitness in the Female Genital Tract due to a Novel Factor under Ovarian Control**

Jessica G. Cole, Nanette B. Fulcher, and Ann E. Jerse

## *Abstract*

The neisserial opacity (Opa) proteins are a family of antigenically distinct outer membrane proteins that undergo phase variable expression. We previously reported Opa-positive variants of *Neisseria gonorrhoeae* strain FA1090 were selected in a cyclical recovery pattern from the lower genital tract of estradiol-treated mice. Here we report the cyclical recovery of Opa-positive gonococci is not dependent on estradiol administration and that consistent with the cyclical recovery pattern, a constitutive Opa-expressing strain had an advantage over an Opa-deficient mutant in the early and late phases of infection. Importantly, we also show the cyclical recovery pattern does not occur in ovariectomized mice, and therefore reproductive hormones may directly or indirectly challenge or benefit *N. gonorrhoeae* during infection. There was no difference in the capacity of Opa-positive and Opa-negative gonococci to adhere to or invade cultured murine cells. Opa-positive variants of a serum sensitive derivative of strain FA1090 showed different degrees of resistance to the bactericidal activity of normal human serum, and fluctuations in vaginal C3 levels in uninfected mice corresponded to the phases of the recovery pattern. However, data from C3-depleted mice did not support complement as the selective factor. We conclude Opa proteins promote persistence of *N. gonorrhoeae* in a surrogate model

of female genital tract infection and *opa* gene phase variation allows gonococci to evade or capitalize on unidentified host factors of the mammalian reproductive cycle. This work reveals an intimate interplay between pathogen and host and is evidence of hormonally-related factors shaping the evolution of bacterial adaptation.

*Note: All of the experimentation in this study was performed by J.G. Cole. The strains FA1090opaA-K and FA1090 opaA-K(B+) were constructed by N.B.Fulcher. At the time of this writing, this manuscript was submitted to Infection and Immunity.*

### *Introduction*

*Neisseria gonorrhoeae* is a Gram-negative pathogen that colonizes the urethra, cervix, pharynx, and rectum of infected individuals. The gonococcus frequently ascends to the upper reproductive tract of females; ascension to the epididymis can also occur in males, and dissemination to the skin and joints via hematogenous spread can occur in both genders (87). The gonococcus has no reservoir outside of humans and therefore has evolved many sophisticated mechanisms for ensuring its survival within humans, including the capacity to vary the expression of surface molecules via high frequency, reversible frame-shift mutations (127). This trait provides a mechanism for evading a specific immune response as well as flexibility in colonizing different niches within the host via functional differences among variant phenotypes.

The neisserial opacity (Opa) proteins are a family of phase variable outer membrane proteins that mediate adherence to and invasion of host cells (reviewed in (131) and resistance to the bactericidal activity of normal human serum (NHS) (20).

Gonococcal strains express 8-10 antigenically distinct Opa proteins that are encoded by separate chromosomal alleles (14, 37, 47). Each *opa* gene independently undergoes phase variation at a rate of  $10^{-3}$ /cell/generation (119) via changes in a pentameric repeated region during replication (130). Bacteria that express no Opa proteins, one Opa protein, or multiple Opa proteins simultaneously result from these reversible mutations. The maintenance of 11-12 *opa* alleles by the gonococcus suggests Opa proteins provide an advantage during infection. This hypothesis is supported by the recovery of mostly Opa-positive urethral isolates from experimentally- (92, 157, 176) and naturally- (90) infected men. Opa expression in the female genital tract appears to be more complex. In an analysis of 104 cervical isolates, predominantly opaque (Opa-positive) colonies were more often isolated pre-ovulation (high estrogen), while mostly transparent (Opa-negative) colonies were isolated prior to or during menses (90). Additionally, in one study Opa-negative variants were isolated from the fallopian tubes, regardless of the stage of the menstrual cycle (49). From these observations, it is hypothesized that certain Opa phenotypes are better adapted for different stages of the reproductive cycle (16, 90). The hypothesis that *N. gonorrhoeae* is affected by hormonally-driven factors is supported by clinical evidence that gonococci are reduced in number or less accessible to cervical culture during the secretory stage of the cycle (high progesterone, low estrogen) (90, 96, 104, 121) and the correlation between the onset of disseminated gonococcal infection (DGI) and menses (5, 74, 85). The factors responsible for the observed associations between Opa phenotype, culture rate, anatomic site, and the menstrual cycle are not known.

Although *N. gonorrhoeae* is restricted to humans, infection of female mice can be established and maintained provided  $17\beta$ - estradiol is administered to promote an estrus-like state. We recently showed that, similar to that which occurs in male volunteers, selection of a subpopulation of Opa-positive gonococci occurred in  $17\beta$ - estradiol-treated mice that were inoculated with a predominantly Opa-negative population. The recovery of Opa-positive variants over time was cyclical with periods of increased percentages of Opa-positive variants occurring early and late in infection. Consistent with distinct subpopulations of bacteria having an advantage *in vivo*, shifts in the predominant Opa phenotype of vaginal isolates were paralleled by fluctuations in the total number of gonococci recovered (165). Here we tested the hypotheses that the cyclical recovery pattern occurs independently of estradiol administration and is due to factors related to the reproductive cycle. We also hypothesized that Opa-positive variants have an adherence advantage or survive better early in infection due to increased resistance to host complement, which is known to be hormonally regulated (76, 112). Our data demonstrate the importance of Opa expression for persistence of *N. gonorrhoeae* in the female genital tract. This work also reveals a novel example of how the evolution of a bacterial species that is confined to mucosal surfaces has been fine-tuned by the mammalian reproductive cycle.

### *Materials and Methods*

**Bacterial strains** *Neisseria gonorrhoeae* strain FA1090 [*porB1b*, streptomycin (Sm) resistant, serum resistant (SR)] was originally isolated from a female patient with

disseminated gonococcal infection. Strain FA1090 has been extensively characterized in male volunteers (35, 92, 160). Strain FA1090<sub>F62por5-8</sub> is a serum sensitive (SS) derivative of strain FA1090, which does not bind human C4B-binding (C4BP) protein due to the replacement of porin loops 5-8 with loops 5-8 of the SS strain F62 (144, 146) (kindly provided by Dr. Sanjay Ram, University of Massachusetts). Frozen stocks containing mostly piliated or nonpiliated colony variants of wild type strain FA1090 or strain FA1090<sub>F62por5-8</sub> were prepared by single colony purification and colony suspension immunoblots using antibodies specific to the Opa proteins of strain FA1090 as described (91, 165). Strains FA1090*opaA-K* and FA1090*opaA-K(B+)* were kindly provided by Dr. Janne Cannon, University of North Carolina. Strain FA1090*opaA-K* is an unmarked recombinant strain derived from wild type strain FA1090 in which all 11 *opa* loci were insertionally inactivated via a selection/counterselection method (97) (*opaA,B,E,G*, and *K*) or single step allelic exchange (*opaC,D,F,I,H*, and *J*) (58). Strain FA1090*opaA-K(B+)* is strain FA1090*opaA-K* into which a phase-locked *opaB* gene that carries alterations in the pentameric repeat region was reintroduced into its native locus (58). The lack of Opa expression by FA1090*opaA-K* bacteria and constitutive expression of OpaB by FA1090*opaA-K(B+)* bacteria were confirmed by colony suspension immunoblots. The LOS phenotypes were similar for all Opa variants of strains FA1090 and FA1090<sub>F62por5-8</sub> tested and for strains FA1090*opaA-K* and FA1090*opaA-K(B+)* based on comparisons of the banding pattern of proteinase K-treated digests (83) fractionated on Bis-Tris 4-12% gradient gels (Invitrogen) followed by silver staining.

**Culture conditions** All *N. gonorrhoeae* strains were cultured at 37°C under 7% CO<sub>2</sub> on GC agar with supplements or GC agar with vancomycin, colistin, nystatin,



trimethoprim sulfate, and streptomycin (VCNTS) for mouse experiments as described (91). Growth curves were performed with agitation at 37°C in supplemented GC broth (GCB) with 0.5 mM NaHCO<sub>3</sub>. At hourly time points, aliquots were diluted in GCB with 0.05% saponin to break up aggregates, and quantitatively cultured overnight on GC agar. Where indicated, water-soluble 17β-estradiol (Sigma) was added to liquid culture at a final concentration of 1000, 100, or 10 pg/mL.

***Tissue culture assay*** ME180 human cervical epithelial cells and HEC1B human endometrial cells were from ATCC. ME180 cells were cultured in McCoy's 5A media supplemented with 10% FBS and 2.2 g/L sodium bicarbonate. HEC1B cells were cultured in DMEM with 10% FBS at 37°C with 5% CO<sub>2</sub>. Two mouse epithelial cell lines, IEC 4.1 (intestinal) and BM1.11 (oviduct) [generously provided by Drs. Harlan Caldwell (Rocky Mountain Laboratories, Hamilton, MT) and Raymond Johnson (Indiana University School of Medicine, Indianapolis, IN), respectively] were cultured as described (151). All tissue culture cells were maintained with 50 µg/mL gentamicin (Gm) during routine passage. For adherence and invasion assays, cells were seeded at a density of  $1 \times 10^5$  cells per well in 24-well tissue culture plates in the absence of antibiotics and infected the following morning with FA1090*opaA-K* or FA1090*opaA-K(B+)* bacteria. Bacteria were suspended to an A<sub>600</sub> = 0.07 in phosphate buffered saline (PBS) and diluted 1:10 in RPMI with 10% FBS and 0.2 µM Fe(NO<sub>3</sub>)<sub>3</sub>. Five hundred microliters ( $3-4 \times 10^6$  CFU) were applied to the cell monolayers to obtain the desired multiplicity of infection (MOI = 30-40). For adherence assays, bacteria were incubated with monolayers for 2 hrs at 37°C under 7% CO<sub>2</sub> and washed 4 times with PBS to remove nonadherent bacteria. Cells were lysed with 0.5% saponin and the number of cell-associated bacteria was

determined by serial dilution and culture. For invasion assays, bacteria were incubated with monolayers for 2 hrs, the cells were washed twice with PBS, Gm (50 µg/ml) was added and the cells were incubated for 1.5 hrs. Monolayers were washed 5 times with PBS, lysed with 0.5% saponin, and the number of internalized bacteria was determined after serial dilution and culture. Results are expressed as the percent of cell-associated bacteria relative to the inoculum (adherence) or as the percent of Gm-protected bacteria relative to the number of adherent bacteria (invasion). All conditions were analyzed in triplicate within an experiment, and each experiment was performed three times.

***Bactericidal assay*** The bactericidal activity of normal human serum (NHS) against colony-purified Opa variants of strain FA1090<sub>F62por5-8</sub> was tested in a microtiter plate assay essentially as described (59). Briefly, NHS (Quidel) or heat-inactivated NHS (HI-NHS) was serially diluted in minimal essential media (MEM) to obtain a final concentration of 0.5-8% serum. Bacteria (18-22 hrs old) were harvested from agar plates, suspended in PBS to an  $A_{600}$  of 0.07 and diluted 1:2000 in MEM. Thirty microliters ( $\sim 1.5 \times 10^3$  CFU) of each suspension were added to each well and the plates were incubated for 1 hr at 37°C with 5% CO<sub>2</sub>. Fifty microliters of GC broth were then added to all wells and a 30 µL aliquot from each well was cultured on GC agar. Colonies were enumerated after 24 hrs, and results are expressed as the percent survival [100 x (the number of CFU recovered from NHS-containing wells divided by the number of CFU from wells with the same concentration of HI-NHS)]. Percents survivals were plotted against NHS dilutions to determine bactericidal<sub>50</sub> titers. In other experiments, the percent survival of different Opa variants following exposure to 3% NHS versus HI-NHS under the same conditions was determined. No killing was observed with HI-NHS.

**Experimental murine infection** Female intact and ovariectomized (Ov<sup>-</sup>) BALB/c mice at 4-6 weeks of age (National Cancer Institute, Frederick, MD) were housed under 12 hour light-dark intervals and allowed food and water *ad libitum*. Ov<sup>-</sup> mice had their ovaries surgically removed at least 14 days prior to the start of the experiment, and all mice were allowed to acclimate to the USUHS animal facility for 10-14 days before the start of an experiment. There are 4 stages of the murine estrous cycle (proestrus, estrus, metestrus, diestrus), which can be differentiated by cytological examination of stained vaginal smears. To promote long-term infection with *N. gonorrhoeae*, diestrus-stage mice were treated with 17 $\beta$ -estradiol either via implantation of a 5 mg, 21-day slow-release pellet (Innovative Research of America) (91) or injection of water-soluble 17 $\beta$ -estradiol (17 $\beta$ -estradiol<sub>ws</sub>) (Sigma) (total dose 1.5 mg) (172). Antibiotics were administered to mice under both treatment regimens as described (91). Mice were inoculated vaginally with bacteria two days after pellet implantation or after the second dose of 17 $\beta$ -estradiol<sub>ws</sub> (91) (172). Estradiol was administered by a slow-release pellet for experiments designed to compare Ov<sup>-</sup> and intact mice, the recombinant strains FA1090*opaA-K* and FA1090*opaA-K(B+)*, and the effect of complement component 3 (C3) depletion. In some experiments, antibiotics but no estradiol were given, and transient colonization was achieved by inoculating mice in the proestrus stage with *N. gonorrhoeae*. In these experiments, soiled litter from a cage of male mice was used to promote synchronization of the cycle (42).

For all mouse infection experiments, mice were inoculated vaginally with 20  $\mu$ l (10<sup>6</sup> CFU) of a filtered bacterial suspension made in PBS as described and vaginal mucus was quantitatively cultured for *N. gonorrhoeae* on GC-VCNTS agar as described (91). In

experiments with wild type FA1090 bacteria, the inocula consisted of mostly nonpiliated colony-purified variants of Opa-negative and OpaB-expressing gonococci harvested from solid GC agar after 20 hr incubation. Suspensions of each variant were combined to obtain ratios of 10-20% OpaB-expressing variants to 80-90% Opa-negative variants as described (165). The Opa phenotypes of 96 colonies isolated from each inoculum and 36 vaginal isolates from each mouse per time point were determined by colony suspension immunoblot (limit of detection for each Opa phenotype, 1% and 3%, respectively) (91, 165). Results are expressed as the fold-increase in Opa-positive variants compared to the inoculum. OpaB and OpaD, and OpaE and OpaK variants were not distinguished in some cases and are referred to as OpaBD and OpaEK variants, respectively.

***C3 capture enzyme-linked immunosorbent assay (ELISA) and complement depletion studies*** To measure vaginal C3 levels in uninfected, estradiol-treated mice, vaginal washes were collected from 4-6 week-old BALB/c mice three days after implantation of a 5 mg 17 $\beta$ -estradiol pellet and then daily for the next 7 days. Antibiotics were also administered to mice as per the infection protocol. Washes were obtained by pipetting 40  $\mu$ L of PBS 5 times in and out of the vagina, and three washes were combined for a total of 120  $\mu$ L from each mouse. Vaginal washes were centrifuged at 13,000 rpm for 3 min to remove cellular debris and the supernatant was frozen at -20°C. The concentration of C3 in vaginal washes was determined by capture ELISA (GenWay Biotech Inc.) as per the manufacturer's specifications. To study the effect of complement on the early phase of infection, cobra venom factor (CVF) (Quidel) was administered intraperitoneally to groups of estradiol-treated mice (20  $\mu$ g CVF per 18-21 g mouse) to deplete C3 the day before bacterial inoculation. A PBS-treated control group was tested

in parallel. To study later time points, estradiol-treated mice were colonized with bacteria for 4 days and then treated with CVF or PBS. For all C3 depletion studies, the number of bacteria recovered from CVF- versus PBS-treated mice each day for three days after treatment was compared. Serum was collected by centrifugation of whole blood obtained by retro-orbital bleeds at 18 and 72 hrs after each CVF dose and the C3 concentration was determined by capture ELISA as above. Serum C3 levels were ~1 mg/mL in normal mice and 90-95% reduced in CVF-treated mice. All experiments were performed at least twice to test reproducibility.

***Animal use assurances*** Animal experiments were conducted in the laboratory animal facility at USUHS, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the USUHS Institutional Animal Care and Use Committee.

***Statistical analyses*** For tissue culture experiments, bactericidal assays and comparisons of C3 levels in vaginal washes an unpaired Student's t-test was used to assess statistical significance. A two-way analysis of variance (ANOVA), followed by an unpaired Student's t-test was used to determine if there was a difference in bacterial load of infected mice or difference in fold changes in recovery of Opa variants. The Log Rank test was used to assess the difference in duration of infection for the recombinant strains FA1090*opaA-K* and FA1090*opaA-K(B+)*. For all analyses,  $p \leq 0.05$  was considered significant.

## Results

### *The cyclical recovery pattern is independent of estradiol treatment*

*N. gonorrhoeae* strain FA1090 expresses 8 antigenically distinct Opa proteins (OpaA, B, C, D, E, F, K and I). Previously we demonstrated that Opa-positive variants of strain FA1090 were selected in 17 $\beta$ -estradiol-treated female BALB/c mice on days 1-3 after inoculation with a bacterial suspension that contained predominantly Opa-negative variants. This early selection phase was followed by increased isolation of Opa<sup>-</sup> variants on days 5-7 (mid-phase) and a subsequent return of Opa-positive variants on days 9-11 (late phase). Changes in the total number of gonococci recovered from vaginal swabs mirrored the shifts in Opa phenotype, with higher numbers corresponding to periods in which Opa-positive gonococci predominated and decreased recovery of bacteria corresponding to the mid-phase (165).

This earlier study was conducted in mice that were treated with 5 mg 17 $\beta$ -estradiol pellets, which release high levels of estradiol for over 2 weeks (172). To determine whether the cyclical recovery pattern occurs in mice that are not under the influence of sustained, nonphysiological concentrations of estradiol, we tested BALB/c mice that were treated with a lower total dose of water-soluble estradiol (estradiol<sub>ws</sub>), which also promotes susceptibility to *N. gonorrhoeae*. Serum estradiol levels in mice treated with estradiol<sub>ws</sub> return to physiological levels within 24 hrs after administration (172). Following challenge of estradiol<sub>ws</sub>-treated mice with a suspension of Opa-negative variants (89%), OpaB variants (11%) and < 1 % of other Opa-positive variants, a 3- to 7-fold increase in Opa-positive variants relative to that in the inoculum was recovered on days 1 and 2 post-inoculation, respectively (Fig. 13A). The mid-phase was observed on

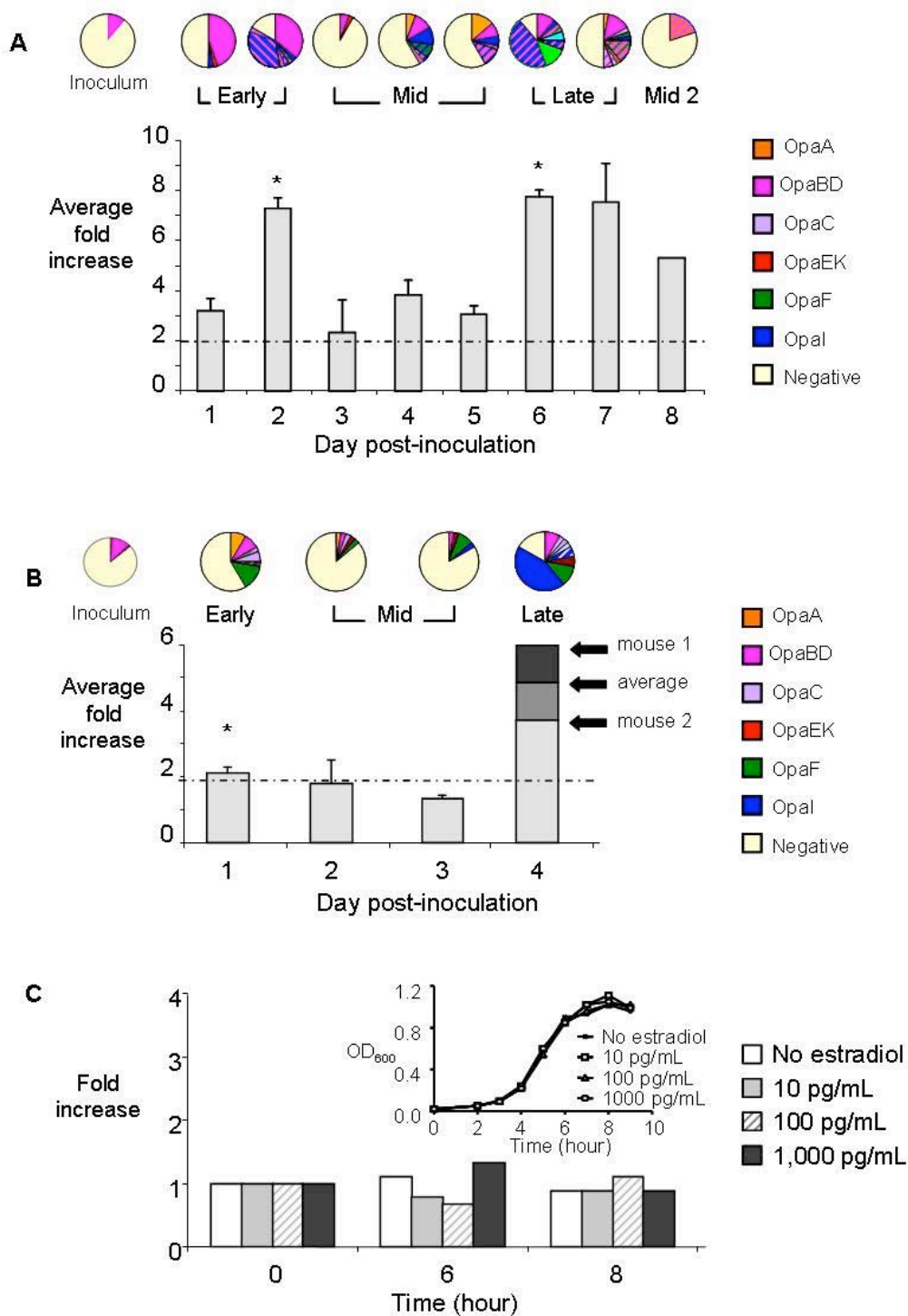
days 3-5 post-inoculation as evidenced by decreases in both the total number of CFU recovered (data not shown) and the relative percentage of Opa-positive variants. This period was followed by an increase in the percentage of Opa-positive variants (late phase). The distribution of Opa phenotypes among vaginal isolates was consistent with the previously observed recovery pattern, with all mice selecting for OpaB early in infection and higher percentages of gonococci that express more than one Opa protein in the late phase. An example of the distribution of Opa variants among vaginal isolates from one representative mouse is depicted by pie charts in Fig. 13A.

We also examined the selection kinetics in mice that were not treated with estradiol. Untreated mice can support transient colonization with *N. gonorrhoeae* when challenged in the proestrus stage of the estrous cycle (42, 95). Combined data from three independent experiments showed that 8 of 11 untreated mice exhibited a 2-fold or greater increase in the recovery of Opa-positive variants compared to the inoculum on day 1 post-inoculation (Fig. 13B). The average percentage of Opa-positive isolates declined to less than 2-fold relative to the inoculum in 5 of 8 mice during the mid phase (days 2 and 3), followed by dramatic increases of ~3.5- and 6-fold in the percent of Opa-positive variants from the two mice that remained colonized for four days. The pie charts in Fig. 13B show the Opa phenotypes of vaginal isolates from an untreated mouse inoculated with a majority of Opa-negative variants (87%) and 11% OpaB, 1% OpaA and 1% OpaEK variants. An early selection for Opa protein expression in this mouse was evidenced by the recovery of 42% Opa-positive isolates on day 1 of infection, with several different Opa-positive variants represented. Following the decrease in the relative

**Figure 13. Estradiol-treated and untreated mice show cyclical Opa recovery during lower genital tract infection but estradiol does not alter gonococcal growth rate or Opa phase variation.** Mice treated with  $17\beta$ -estradiol<sub>ws</sub> (A), or no estradiol (B) showed cyclical Opa recovery after inoculation with a suspension of predominantly Opa-negative variants. Pie charts show the Opa phenotype of a representative mouse from each experiment with colors corresponding to the different Opa proteins of strain FA1090. Variants that express multiple Opa proteins are indicated by a striped pattern. The average fold-increase in Opa-positive variants among vaginal isolates relative to the inoculum is displayed in the histograms. The dashed line indicates selection, which was defined as a >2-fold increase in Opa-positive variants compared to the inoculum. Bars indicate SEM and asterisks indicate  $p < 0.03$  compared to the mid-phase, which was defined as days 3-5 in panel A and day 3 for panel B. Results shown in panel A are from one experiment with 6 mice inoculated with 89% Opa-negative and 11% OpaB variants and are representative of results from two independent experiments ( $n = 5-6$  mice per experiment). Results shown in panel B are combined data from 3 experiments in which untreated mice were colonized for 1-4 days. The average fold-increase was based on results from 11 mice on day 1, 5 mice on day 2, 3 mice on day 3 and 2 mice on day 1. (C) Recovery of Opa-positive isolates from liquid culture at different stages of growth expressed as fold-increase compared to the inoculum. GCB cultures with no estradiol or 10, 100, or 1,000 pg/mL  $17\beta$ -estradiol<sub>ws</sub> were inoculated with a bacterial suspension that consisted of 75% Opa-negative variants. The Opa phenotypes of 96 colonies isolated from aliquots taken at 0 hrs, or 36 colonies at 6 and 8 hrs were determined. No change in Opa phenotype was observed under any condition analyzed.



Figure 13. Estradiol-treated and untreated mice show cyclical Opa recovery during lower genital tract infection but estradiol does not alter gonococcal growth rate or Opa phase variation.



number of Opa-positive variants on days 2 and 3, 83% of the isolates on day 4 expressed one Opa protein (solid colors) or several Opa proteins simultaneously (striped patterns) Opa proteins, with the majority of isolates expressing OpaI (dark blue). A similar pattern was observed in the second mouse that was colonized through day 4, although the predominant Opa phenotype of isolates recovered on day four was OpaEK (69% of isolates) (Fig. 13B). These results are consistent with the three phases of recovery seen in estradiol-treated mice and show Opa-expressing variants have a clear advantage *in vivo* over time.

The early selection for Opa-positive gonococci in untreated mice was not as strong as in estradiol-treated mice and the timing of each phase of the recovery pattern differed in untreated, pelleted and estradiol<sub>ws</sub>-treated mice. We therefore tested whether estradiol directly selects for Opa-positive variants *in vitro*. We found no change in the percentage of Opa-positive variants relative to that in the starting inoculum when a suspension of primarily Opa-negative gonococci was cultured in broth that contained 17 $\beta$ -estradiol at concentrations that are similar to the peak serum estradiol levels in pelleted, estradiol<sub>ws</sub>-treated and untreated mice (172) (Fig. 13C). We conclude that there is an endogenous host factor(s) in the lower genital tract of female mice that selects for distinct subpopulations of different Opa phenotypes in a cyclical pattern. Estradiol itself is not directly responsible for the observed pattern, although this factor appears to be modulated *in vivo* by the administration of 17 $\beta$ -estradiol.

### ***The cyclical recovery pattern is dependent on the ovaries***

Cyclical fluctuations in the number of gonococci recovered from mice and the Opa phenotype of vaginal isolates suggests hormonal factors challenge *N. gonorrhoeae*

during murine infection. Ovariectomized (Ov<sup>-</sup>) mice can be used to study different aspects of gonococcal infection in the absence the reproductive cycle, and can be infected with *N. gonorrhoeae*, although 17 $\beta$ -estradiol and antibiotic treatment is required (94). To test the role of the reproductive cycle in the cyclical recovery pattern, we inoculated estradiol-treated intact and Ov<sup>-</sup> mice with a suspension of mostly Opa-negative variants and determined the number of gonococci recovered and the Opa phenotype of vaginal isolates over time. Selection for Opa-positive variants occurred in both intact and Ov<sup>-</sup> mice on day 1 of infection. A cyclical recovery was then observed in intact mice as expected, with increased percentages of Opa-positive variants (Fig. 14A) corresponding to periods of increased (early and late phase) CFU recovered (Fig. 14B). In contrast, selection for Opa-positive gonococci was maintained in Ov<sup>-</sup> mice (Fig. 14A), and the number of gonococci recovered stayed at a constant level throughout the 15 day experiment (Fig. 14B). We conclude that the changes in Opa selection and colonization load that characterize the cyclical recovery pattern are influenced directly or indirectly by ovarian factors.

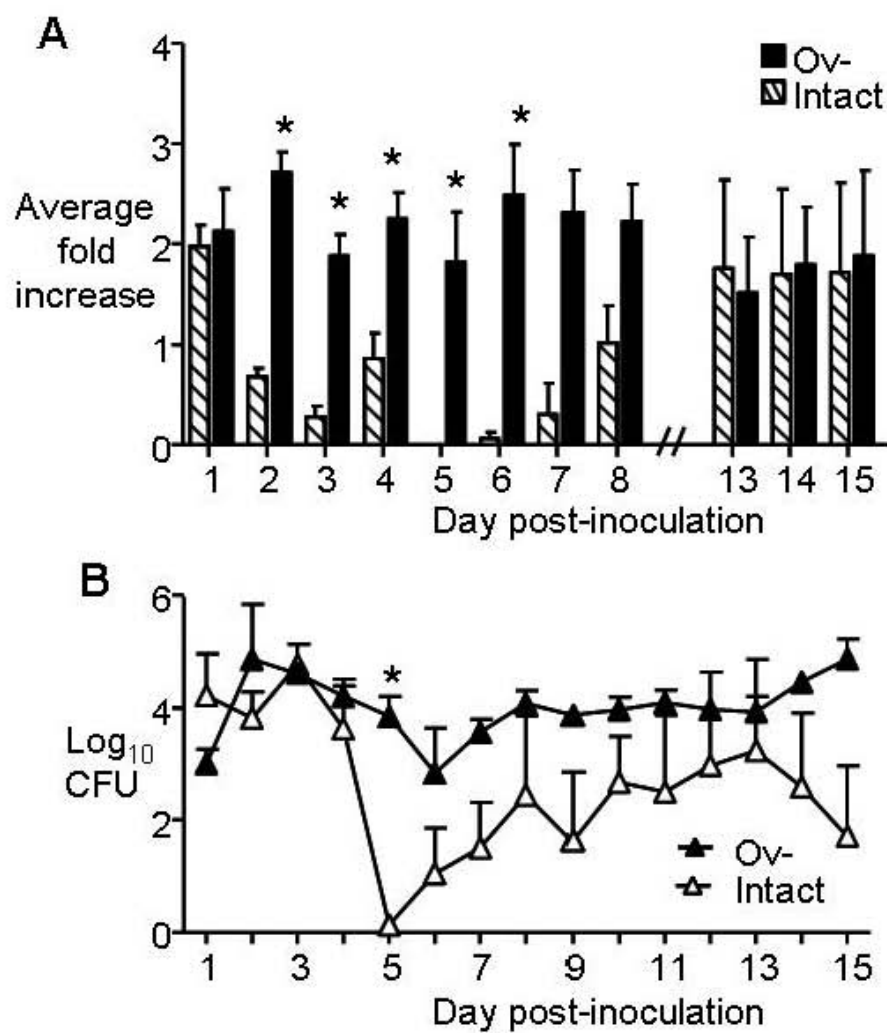
### ***Opa proteins provide an early and late colonization advantage***

Due to the strong selection for an Opa-positive phenotype in the early phase of infection, we hypothesized that Opa protein expression confers an advantage soon after entering the vagina and that Opa-negative gonococci do not colonize mice as well as do Opa-expressing gonococci. To test this hypothesis, we utilized a genetically defined mutant of strain FA1090 in which all the *opa* genes were inactivated (FA1090*opaA-K*) and a derivative of this Opa-deficient mutant into which a phase-locked *opaB* gene was reintroduced (FA1090*opaA-K(B+)*). The growth kinetics of strains FA1090*opaA-K* and

**Figure 14. Normal (intact) but not Ov<sup>-</sup>, mice show the cyclical recovery pattern.**

Groups of intact or Ov<sup>-</sup> mice were implanted with 5 mg 17 $\beta$ -estradiol pellets and inoculated with 10<sup>6</sup> CFU of a suspension containing 70% Opa-negative variants and 23% OpaB, 3% OpaC, 2% OpaF, and 1% OpaF and OpaI. The Opa phenotype of vaginal isolates and number of gonococci recovered was determined over time. **(A)** Opa phenotype of vaginal isolates. Selection for Opa expression, which was defined as 2-fold increase in the percentage of Opa-positive variants recovered compared to the inoculum, occurred in both groups on day 1, but the subsequent loss and re-emergence of Opa-positive variants in intact mice (hatched bars) did not occur in Ov<sup>-</sup> mice (solid bars). **(B)** Total number of CFU recovered over time. Fluctuations in the total number of CFU recovered from vaginal swab suspensions was observed in intact mice (open triangles) as reported previously (165), with the dramatic decline that characterizes the mid-phase occurring on days 5-8. Ov<sup>-</sup> mice showed a more uniform recovery of viable gonococci over time (solid triangles). An asterisk is used to denote a significant difference ( $p < 0.05$ ) between the number of gonococci recovered from each group on day 5 and horizontal bars indicate SEM. In the experiment shown, there were 3 mice per group, except for intact mice on days 11-15, for which results from 2 mice are shown. Results were consistent in a second experiment with 3-5 mice per group.

Figure 14. Normal (intact) but not Ov<sup>-</sup> mice show the cyclical recovery pattern.

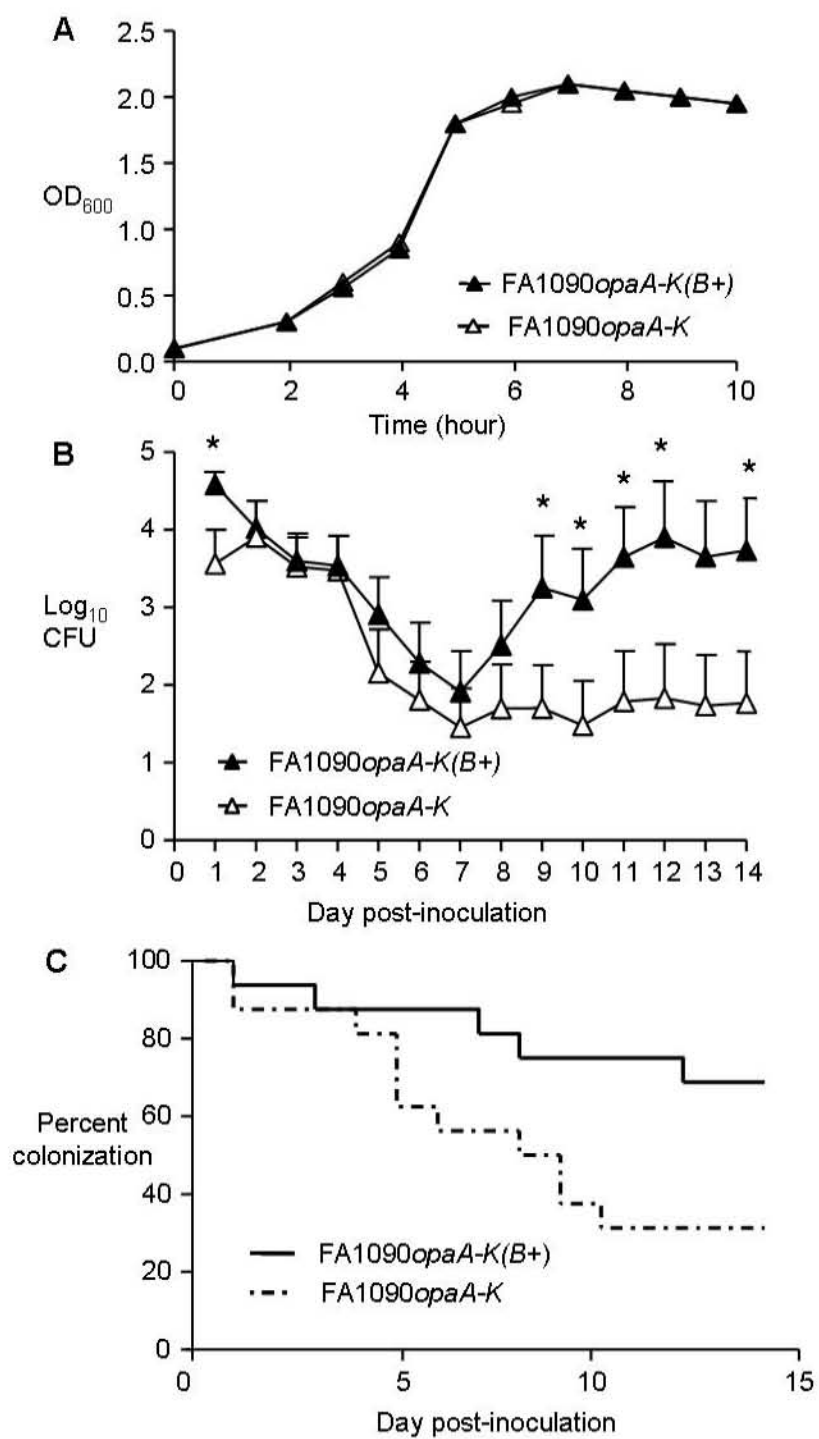


FA1090*opaA-K(B+)* were similar when cultured in liquid media (Fig. 15A). We therefore next compared the capacity of these two strains to infect estradiol-treated mice. Mice were inoculated with  $10^6$  CFU of either strain and cultured over time. Consistent with Opa expression conferring a colonization or survival advantage early during infection, a higher number of bacteria was recovered from mice inoculated with the OpaB-expressing strain on day 1 compared to mice inoculated with the Opa-deficient strain ( $p < 0.05$ ) (Fig. 15B). A decrease in the number of bacteria recovered occurred in both groups on days 4-7 (mid-phase), and a steady increase in the recovery of OpaB-expressing bacteria began on day 8 and lasted through day 14, which is temporally consistent with the late phase. The number of gonococci recovered was significantly higher from mice inoculated with the OpaB-expressing strain versus the Opa-deficient mutant on days 9-12 and 14 ( $p < 0.05$ ). Furthermore, in contrast to the OpaB-expressing strain, the Opa-deficient strain was less able to recover from the mid-phase. Colonization curves demonstrate that strain FA1090*opaA-K(B+)* is more fit compared to strain FA1090*opaA-K* in terms of establishing persistent infection, with 69% and 31% of mice infected on day 14, respectively ( $p = 0.037$ ) (Fig. 15C).

These results are the first demonstration of the importance of Opa proteins using defined mutants in an *in vivo* model. We conclude that i.) OpaB provides an early advantage during infection of female mice, ii.) the factors that are responsible for the decreased recovery of viable bacteria in the mid-phase appear Opa-independent, and iii).

**Figure 15. An OpaB-expressing strain colonizes 17 $\beta$ -estradiol treated mice better than does an Opa-deficient mutant.** The Opa-deficient strain FA1090*opaA-K* and the OpaB-expressing strain FA1090*opaA-K(B+)* were compared for differences in growth in liquid culture and for the capacity to colonize estradiol-treated mice. **(A)**  $A_{600}$  readings of GCB cultures inoculated with FA1090*opaA-K* or FA1090*opaA-K(B+)* bacteria over time. **(B)** Average number of CFU recovered on vaginal swabs following inoculation of estradiol-treated mice with  $10^6$  CFU of Opa-deficient (open triangles) or OpaB-expressing bacteria (closed triangles). Horizontal bars indicate the SEM and asterisks denote a significant difference between groups ( $p < 0.05$ ; Student's *t* test). **(C)** Percentage of mice colonized at each day following bacterial inoculation with strains FA1090*opaA-K* versus FA1090*opaA-K(B+)*. Significantly more mice were culture-positive following inoculation with the constitutive OpaB-expressing strain (solid line) compared to mice inoculated with the Opa-deficient strain (broken line) on days 9-12 and day 14 ( $p = 0.037$ ; log rank test). Results in panels A and B are from two combined experiments with each symbol representing 16 mice.

Figure 15. An OpaB-expressing strain colonizes 17 $\beta$ -estradiol treated mice better than does an Opa-deficient mutant.





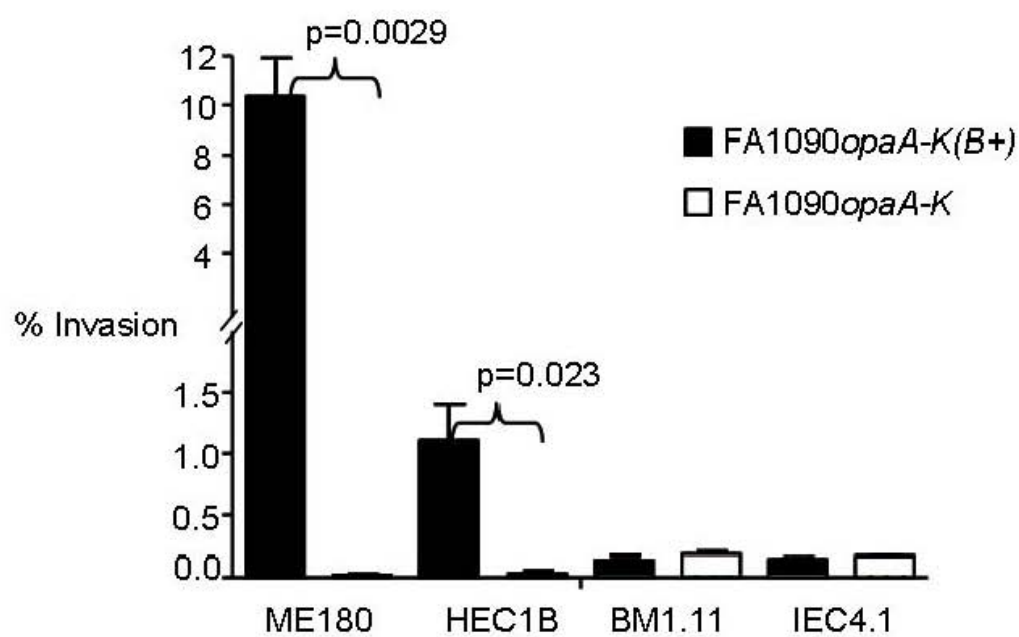
Opa-deficient gonococci are less able to recover from the mid-phase, presumably due to a factor that appears later in infection which gives Opa-expressing gonococci a selective advantage *in vivo*.

***OpaB does not mediate invasion of murine tissue culture cells***

Members of the human carcinoembryonic antigen cellular adhesion molecule (CEACAM) family serve as receptors for Opa-mediated uptake of gonococci by epithelial cells, although other Opa receptors have been described (reviewed in (131)). The presence of an Opa-specific colonization receptor on murine epithelial cells could explain our results, and we therefore, compared the capacity of the OpaB-expressing FA1090*opaA-K(B+)* bacteria and Opa-deficient strain FA1090*opaA-K* bacteria to adhere to and invade murine IEC4.1 (intestinal) and BM1.11 (oviduct) cells. ME180 cells, which are a CEACAM-expressing human endocervical cell line, and HEC1B cells, a human CEACAM-negative endometrial cell line that supports Opa-dependent invasion (177), were used as controls. Both human cell lines supported Opa-dependent invasion as evidenced by significantly more of the OpaB-expressing strain recovered following Gm treatment than the Opa-deficient strain (Fig. 16). Ten-fold more internalized OpaB-expressing gonococci were recovered from ME180 cells compared to HEC1B cells, a result that is consistent with the efficacy by which Opa protein-CEACAM interactions mediate uptake. In contrast to the human cells, the two murine cell lines did not support Opa-dependent invasion. We also found no difference in the capacity of the Opa-positive versus Opa-negative strain to adhere to IEC4.1 or BM1.11 cells (data not shown).

**Figure 16. Opa-mediated invasion occurs in immortalized human, but not murine tissue culture cells.** The capacity of strains FA1090*opaA-K(B+)* and FA1090*opaA-K* to invade human (ME180, HEC1B) and mouse (IEC4.1, BM1.11) cells was compared using the Gm protection assay. A higher number of FA1090*opaA-K(B+)* CFU was recovered from both human cell lines after Gm treatment compared to FA1090*opaA-K*, with 10-fold more internalization of the Opa-expressing strain by the CEACAM-expressing ME180 cells compared to CEACAM-negative HEC1B cells. No difference in invasion was detected in either mouse cell line for FA1090*opaA-K(B+)* or FA1090*opaA-K*. No difference in the number of cell-associated gonococci was observed between the two strains for any cell line (data not shown). Percent invasion was calculated relative to the number of adherent bacteria after 2 hrs and before Gm treatment (data not shown). Bars indicate SEM from three independent wells. Similar results were obtained in three independent experiments.

Figure 16. Opa-mediated invasion occurs in immortalized human, but not murine tissue culture cells.



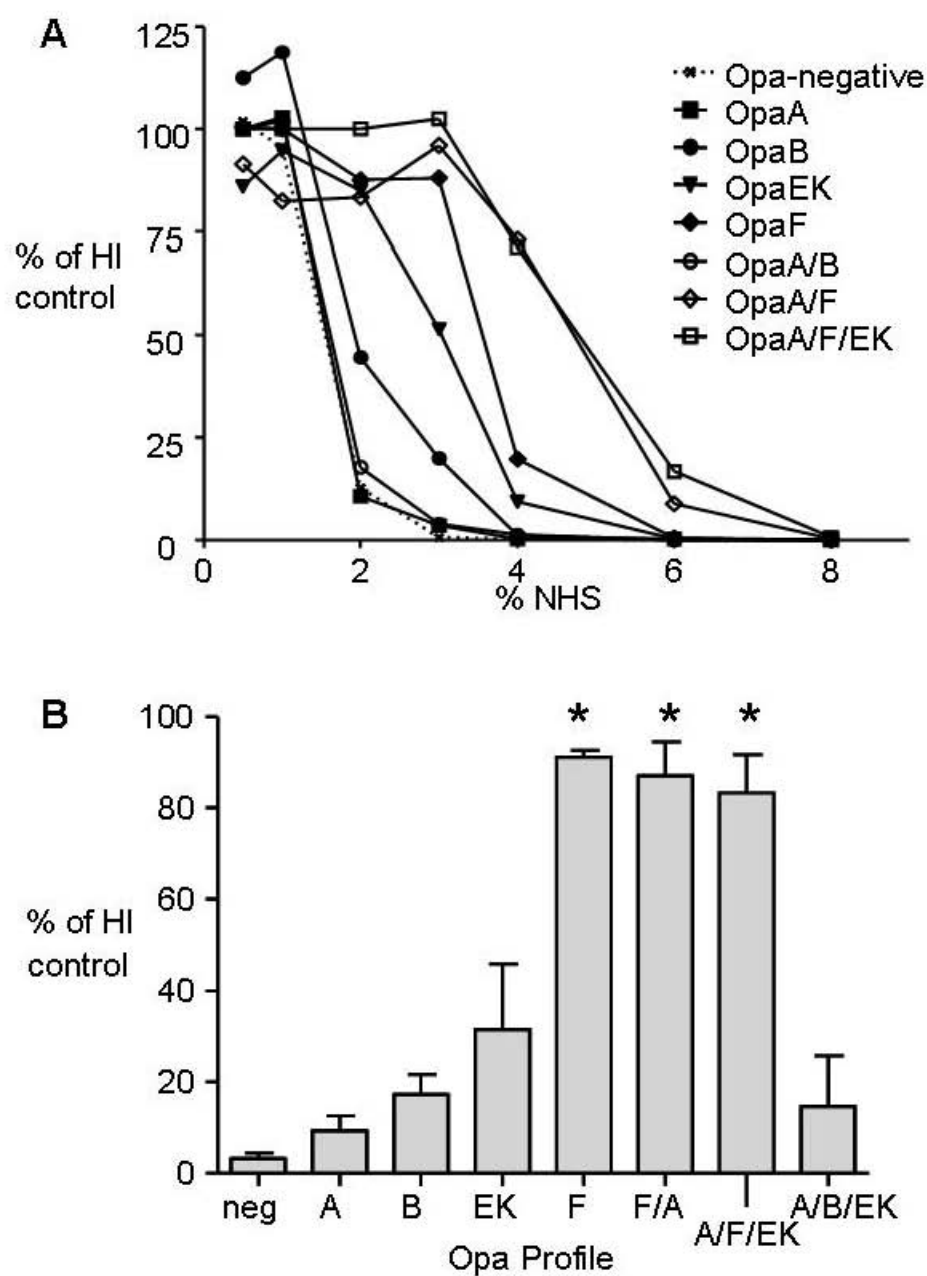
These results are inconsistent with the *in vivo* advantage exhibited by strain FA1090*opaA-K* (*B+*) as a result of OpaB-mediated adherence or invasion, although we cannot rule out the possibility that Opa-specific colonization receptors may be expressed during infection.

### ***C3 depletion does not affect the early selection phase***

Hormonal regulation of complement levels in the female genital tract is well established in humans (76) and mice (112), and Bos *et al.* (20) reported Opa-positive variants of *N. gonorrhoeae* strain MS11 were more resistant to the bactericidal activity of low concentrations of NHS. Strain FA1090, in contrast to strain MS11, is highly resistant to NHS and our attempts to demonstrate Opa-mediated increases in serum resistance in strain FA1090 were unsuccessful. However, because the high level of serum resistance in strain FA1090, which is porin-mediated, is host-restricted (132), and thus does not occur during murine infection, it seemed prudent to further investigate the role of Opa proteins in evading host complement. Murine complement is very labile and thus mouse serum cannot be used to test the bactericidal activity of mouse complement at low concentrations such as that which would be present at mucosal surfaces. We therefore compared the NHS sensitivity of Opa variants of strain FA1090<sub>F62por5-8</sub>, which is a serum-sensitive derivative of strain FA1090 that does not bind human C4BP due to an altered *porB* gene. The bactericidal<sub>50</sub> titers of NHS against Opa variants strain FA1090<sub>F62por5-8</sub> ranged from 1.8% (Opa-negative, OpaA, OpaA/B), 2.2% (OpaB), 3.4% (OpaEK), 3.8% (OpaF), and ca. 5.1% (OpaA/F and OpaA/F/EK) when results from assays in which 2-4 different variants of strain FA1090<sub>F62por5-8</sub> were tested were compared (Fig. 17A). To be able to compare the sensitivity of several variants in the same experiment, we tested a

**Figure 17. Some Opa proteins increase resistance to NHS in vitro.** The bactericidal activity of NHS was tested against different Opa variants of the serum sensitive derivative FA1090<sub>F62por5-8</sub>. **(A)** Percent survival of each variant tested with respect to the dilution of NHS tested. Representative data from several independent assays shows a shift in the bactericidal<sub>50</sub> titer compared to Opa-negative variants for variants that express OpaB, OpaEK, OpaF, OpaA/F, and OpaA/F/EK, but not OpaA or OpaA/B. **(B)** Percent survival of different Opa variants following incubation in 3% NHS. Results are expressed as the average percent survival when data from at least three independent experiments are combined. Horizontal bars denote the SEM and asterisks indicate significance at a level of  $p < 0.0001$ . Percent survival is the number of CFU recovered from wells with NHS divided by the number recovered from wells with the same concentration of heat-inactivated (HI) NHS.

Figure 17. Some Opa proteins increase resistance to NHS in vitro.



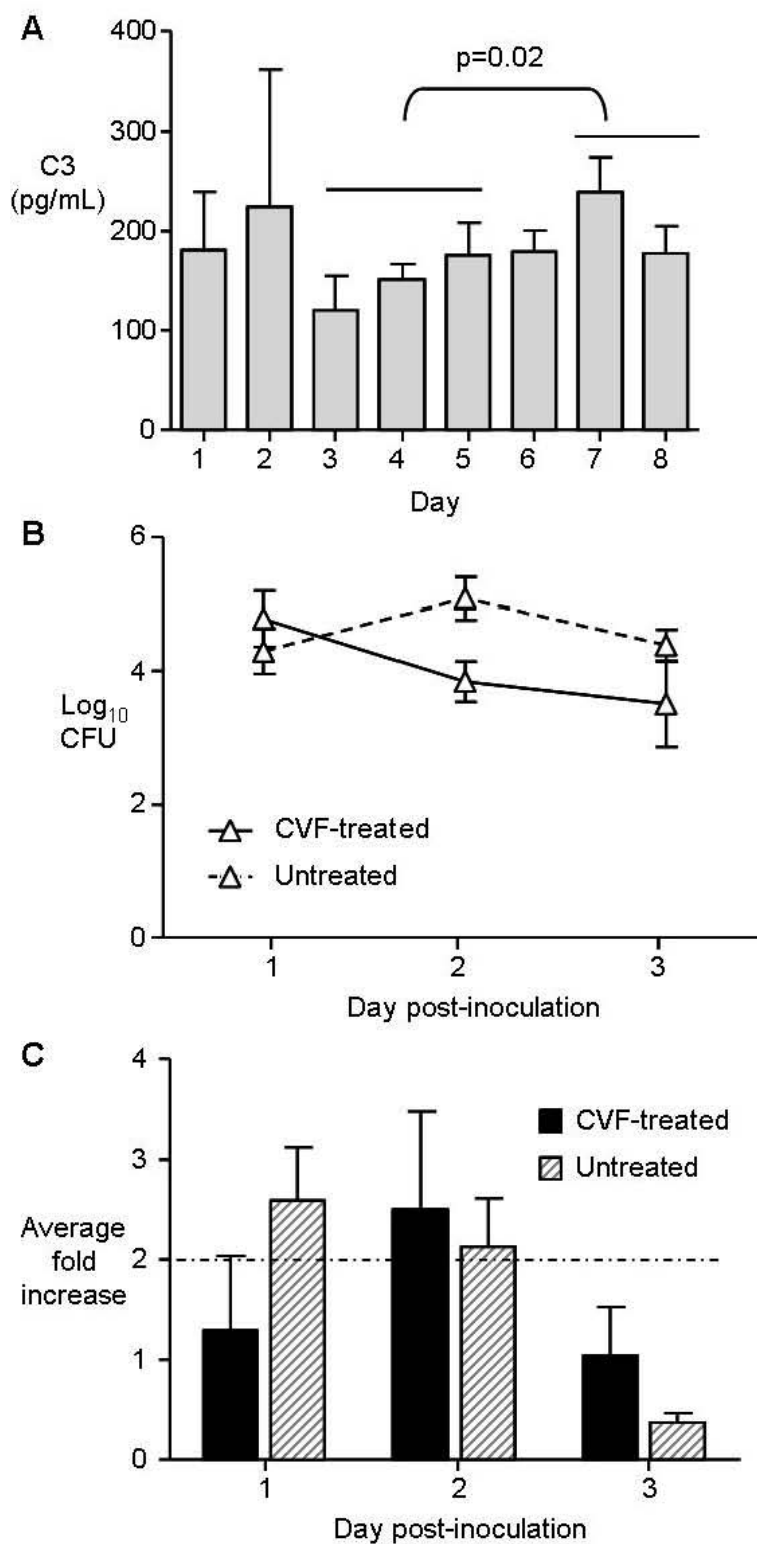
single concentration of NHS, 3%, which fell in the linear region of the bactericidal curves. The percent survival of different Opa variants following incubation in 3% NHS showed a similar gradient in resistance, although only OpaF, OpaA/F, and OpaA/F/EK were statistically more resistant to NHS compared to Opa<sup>-</sup> variants when combined results from at least three independent experiments were compared ( $p < 0.001$ ) (Fig. 17B). The percent survival of OpaB variants and OpaEK variants, which are commonly selected during murine infection, was not significantly different than of Opa-negative gonococci when this concentration of serum was tested.

To further analyze the role of complement as a selective factor for Opa expression, we measured the level of C3 in vaginal washes from uninfected, estradiol-treated mice. The average C3 concentration was higher on the days that correspond to days 1 and 2 of infection versus days 3-5 in each of two independent experiments, although there was much variability among mice and this difference was not statistically significant. However, significantly lower levels of vaginal C3 were detected between the time points that correspond to the mid-phase (days 3-5) versus the late phase (days 7-8) ( $p=0.02$ ) (Fig. 18A). We next treated mice with cobra venom factor (CVF) to deplete C3 on the day prior to inoculation with the Opa-deficient strain, with the prediction that the recovery of the mutant would increase in the early phase of infection if Opa-negative gonococci were susceptible to complement *in vivo*. We found no difference in the number of CFU recovered from CVF-treated versus PBS-treated mice on days 1-3 (Fig. 18B). We also challenged CVF- and PBS-treated mice with a bacterial suspension of wild type gonococci that contained 80% Opa-negative variants. A 2-fold increase in Opa-

**Figure 18.** Mucosal C3 levels fluctuate over time but complement is not responsible for the early selection of Opa-positive gonococci. The role of complement in the cyclical recovery pattern was examined indirectly by measuring C3 levels in vaginal washes and directly by performing infection studies in C3-depleted mice. **(A)** Concentration of C3 in vaginal washes from uninfected mice that were treated with 5 mg 17 $\beta$ -estradiol slow-release pellets. A significantly higher concentration of C3 was present in vaginal washes from days 7-8 (late phase) compared to days 3-5 (mid-phase). Results are combined from two independent experiments with  $n = 10-13$  mice per day. **(B)** Effect of C3 depletion on the recovery of strain FA1090 $opaA-K$ . There was no difference in average number of Opa-deficient gonococci recovered from mice treated with CVF (solid lines) or PBS (dashed lines) on days 1-3 of infection. Results are from a single experiment with 7 mice per group and similar results were obtained in a second independent experiment. **(C)** Recovery of Opa-positive variants in mice treated with PBS versus CVF to deplete C3 the day prior to bacterial inoculation. Mice were challenged with a predominantly Opa<sup>-</sup> (80%) suspension of wild type FA1090 gonococci and the percentage of Opa-positive variants among vaginal isolates on days 1-3 post- inoculation was determined. Selection for Opa expression, as defined as a 2-fold increase relative to the inoculum (dotted line) occurred in both groups on day 1 or two post-bacterial challenge. Results shown are from a single experiment in which  $n = 3-4$  mice per group. In all panels, horizontal bars represent the SEM.



Figure 18. Mucosal C3 levels fluctuate over time but complement is not responsible for the early selection of Opa-positive gonococci.



positive variants occurred on days 1 or 2 post-inoculation in both groups, a result that further suggests complement does not mediate the early recovery phase (Fig. 18C).

Although C3 levels were decreased in untreated mice at time points that correspond to the mid-phase (Fig 18A), we also investigated the role of complement in the decreased recovery of gonococci that we see during this phase of infection. Both CVF-treated and untreated mice inoculated with wild type gonococci showed a decrease in the percentage of Opa-positive gonococci recovered on day 3, which is consistent with the beginning of the mid-phase. However, serum levels of C3 increase within 3 days of CVF administration and so we designed a separate experiment to investigate the role of C3 in the mid-phase. Groups of estradiol-treated mice were colonized with the OpaB-expressing strain or the Opa-deficient mutant for 4 days and then given CVF or PBS and cultured for 3 more days. A drop in the number of recoverable CFU occurred in all groups starting on days 3-4 and there was no difference in the number of bacteria recovered from either group on days 5-8 for either strain (data not shown). From these studies, we conclude complement is not responsible for the characteristic decrease in vaginal CFU that occurs in the mid-phase of infection.

In summary, in the absence of porin-mediated resistance in a highly serum resistant strain, some Opa proteins, either alone or in conjunction with others, confer increased resistance to concentrations of NHS that reasonably mimic the concentration of complement at mucosal surfaces. Whether the shift that we observed in bactericidal<sub>50</sub> titers is biologically significant is not known; however, at one concentration tested, 3%, the percent survival of OpaB variants, which are selected *in vivo*, was not significantly more resistant than Opa-negative bacteria. Additionally, data from CVF-treated mice

were not consistent with complement mediating the early selection for Opa-positive gonococci that occurs during murine infection. We conclude that while complement may contribute to the selection of Opa variants during infection, it is not the sole factor that mediates the early selection of Opa-positive variants during murine infection.

### *Discussion*

The capacity to maintain reservoirs of bacteria that differ in the expression of surface molecules such as the Opa proteins is a major adaptation mechanism utilized by *N. gonorrhoeae*. Here we present experimental evidence in support of earlier speculations that *opa* gene phase variation is a mechanism by which *N. gonorrhoeae* adapts to hormonally driven changes in women of reproductive age. While selection for Opa expression occurred early in infection of mice that lack ovaries, it was maintained at a constant level and did not undergo a cyclical recovery as that which occurs in mice with intact reproductive systems. We also showed that estradiol administration, which is used to promote susceptibility to *N. gonorrhoeae* in mice, is not required for the cyclical recovery of Opa-positive gonococci.

A second important finding was our demonstration that Opa proteins promote persistence in the lower genital tract of a surrogate female host. Mice infected with a constitutive OpaB-expressing strain remained colonized longer and at higher levels than an isogenic Opa-deficient strain. Additionally, the Opa-positive strain was better able to recover from the period of reduced isolation that occurs 5-7 days post-inoculation in mice treated with slow-release estradiol pellets. The culture-negative “window” that

characterizes the mid-phase is frequently seen during experimental murine infection (91), and like Opa selection, appears to be dependent on reproductive hormones due to its absence in *Ov<sup>-</sup>* mice. This phenomenon appears to mimic the hormonally related changes in cervical colonization loads that have been documented in women with gonorrhea. Women with gonorrhea were more often culture-positive in the proliferative stage of the menstrual cycle and culture-negative in the mid-secretory phase (96, 121, 178). In one study, 4 infected women with normal menstrual cycles were hospitalized and left untreated so the recovery of *N. gonorrhoeae* could be followed over the course of a full cycle. Positive cultures correlated strongly with the proliferative and ovulatory phases, and all 4 women had 5-6 consecutive negative cultures during the secretory phase. In each case, cultures became positive again in the pre-menstrual phase or during menses (104). The factors responsible for these culture-negative periods are not known; here we ruled out complement as mediating the reduced recovery of gonococci that occurs during the mid-phase of infection in the mouse model.

To our surprise, while studies with wild type gonococci suggest Opa-negative variants have an advantage during the mid-phase, the mid-phase appeared to be Opa-independent when defined mutants were tested. Based on variations in the colony opacity of human cervical isolates, which correlate with different stages of the menstrual cycle (90), we hypothesize the forces that select for the Opa-negative phenotype during the mid-phase may be subtle and only detectable when relative numbers of Opa-negative and Opa-positive gonococci are compared within the same mouse. We also cannot rule out the possibility that bacterial invasion into tissue may contribute to the decrease in recoverable bacteria during the mid-phase. Tissue remodeling in preparation for

implantation or sloughing should fertilization not occur is orchestrated by hormonally-regulated proteases, which may directly select for certain Opa variants (16, 175) or open an avenue for bacterial invasion. Gonococci are seen within vaginal and cervical tissue as early as two days post-infection in the mouse model (172), and the effect of tissue invasion on the cyclical recovery pattern has not yet been tested. We attempted to use polarized HEC1B cells to determine whether Opa proteins mediate invasion across an epithelial cell layer in the absence of CEACAMs. However, the HEC1B monolayers did not maintain their integrity, as measured by a horseradish peroxidase-leakage assay (189) for longer than 2 hrs, which was an inadequate time period to evaluate this question.

The identity of the host factor(s) responsible for selection for Opa-positive gonococci *in vivo* is not known. Opa-mediated adherence and invasion is well characterized at the molecular level; however, the role of Opa proteins as colonization factors in mice and urethral infection of male volunteers remains unclear. The tissue distribution of the major class of Opa adherence receptors, the human CEACAMs, does not correlate with the predominant sites of gonococcal infection in humans. For example, limited or no CEACAM expression was detected on primary male urethral epithelial cells (75), and some normal cervical and fallopian tube cells (177). Mice express only CEACAM1, which differs from human CEACAM1 in several critical amino acids (185). Consistent with the prediction that Opa-CEACAM interactions are host-restricted, we found no difference in the capacity of Opa-positive and Opa-negative gonococci to adhere to or invade murine cells. We also found no evidence of Opa-mediated adherence to an unidentified receptor on murine cells and thus hypothesize selection for Opa-positive variants in mice must be due to functions that are unrelated to the role of Opa

proteins as colonization factors. Some Opa proteins mediate uptake by epithelial cells via binding to heparin sulfate proteoglycans (HSPG) (131) and HSPG is present on genital tract tissues (77). However, only OpaI of strain FA1090 binds HSPG (58), and although OpaI can be selected following inoculation of mice with mostly Opa-negative variants (165), competition experiments in our laboratory between OpaI and OpaB variants did not show preferential selection for OpaI during infection (Cole and Jerse, unpublished observation). These results bring into question the importance of Opa-HSPG interactions during murine infection.

Our favored hypothesis was that host complement is responsible for selection for Opa-positive variants early and late during infection based on the known hormonal regulation of complement in females (76, 112) and the demonstration of Opa-mediated serum resistance in another strain. Serum resistance is a complicated phenotype in *N. gonorrhoeae* due to the existence of several mechanisms that are not expressed by all strains. Although the expression of some Opa proteins in a serum sensitive derivative of FA1090 confers increased resistance to NHS, this is not a characteristic of all Opa proteins, including OpaB and OpaEK, which showed cyclical recovery here or in our previous studies (165). In studies with strain MS11, expression of certain phase variable species of lipooligosaccharide (LOS) was phenotypically dominant over Opa proteins in mediating serum resistance (20). We saw no difference in the LOS species produced by the Opa variants tested here or the Opa-deficient and constitutive OpaB-expressing strains (data not shown), however, and thus our results are unlikely to be confounded by differences in LOS phenotype. We also showed depletion of C3 to a level equal to 90-95% of that found in untreated mice does not alter selection for Opa expression early

during infection. The use of complement-deficient mice would be a more stringent test of our hypothesis; however, C3- and C5-deficient mice are only available in the C57/BL6 background. We have not yet characterized Opa expression in C57/BL6 mice, and predict it may be different due to differences in the innate response to *N. gonorrhoeae* in BALB/c versus C57/BL6 mice (M. Packiam *et al.*, submitted).

Other effectors in the dynamic environment of the female genital tract may play a selective role *in vivo*. Hormonally regulated changes in the levels of antimicrobial peptides (102), lactoferrin (33), progesterone, proteases, protease inhibitors, pH, mucus viscosity, and the number and composition of commensal flora have been documented. One or more of these factors may differentially select for Opa-positive or Opa-negative gonococci, or a combination of factors may be required, such as the reported synergy between lactoferrin, lysozyme and serine leukocyte protease inhibitor (SLPI) against *Escherichia coli* (167). Future investigations into the host factors that are responsible for cyclical recovery patterns we describe here should further elucidate the role of *opa* gene phase variation in gonococcal adaptation to the female host.

### *Acknowledgments*

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## Chapter 4: Discussion

The Opa proteins of *N. gonorrhoeae* are a family of phase variable outer membrane proteins involved in adherence and invasion of epithelial cells, resistance to complement-mediated killing, and immunosuppression of the host. The first major focus of this work was to evaluate the potential of conserved Opa loops as vaccine candidates. We characterized the effector functions and *in vivo* protective capacity of antibodies induced by cyclic or linear peptides that correspond to the predicted surface-exposed loops. These results are presented in Chapter 2. Chapter 3 summarizes results from the second aim of this dissertation, which was conducted to investigate the interplay between the female host and the gonococcus as it relates to Opa protein expression.

### *Characterization of antibodies that target conserved Opa loops*

Natural infection with *N. gonorrhoeae* does not generate a protective immune response, and the reasons underlying this lack of protection are not known. There are currently no correlates of protection defined for *N. gonorrhoeae*. However, correlates of protection against *N. meningitidis*, which are based on antibody function, are assumed to be applicable to gonococcal vaccines. This work was undertaken to investigate the potential of Opa proteins as vaccine candidates and to define protective correlates for antibody-based protection in an animal model.

Here, we characterized antibodies that target the conserved Opa loops with the long term goal of generating Opa-specific antibodies that, when administered topically, prevent gonococcal infection. There are four predicted surface-exposed loops in a mature



Opa protein (117) with the first and fourth loops being semi- and highly conserved, respectively. Most antibodies developed against Opa proteins during natural infection, or following immunization with purified Opa proteins, target the hypervariable loops 1 and 2 (HV<sub>1</sub> and HV<sub>2</sub>) (44, 196). Antibodies developed to the HV loops during infection are not protective; therefore, we chose to develop antibodies that target peptides corresponding to the two conserved loops. The predicted amino acid sequences for the semi-variable (SV) loop from all Opa proteins expressed by strains FA1090 and MS11 were analyzed. There was slightly more diversity within the SV loop sequences from strain MS11 compared to strain FA1090, and a target peptide that corresponds to the OpaA and OpaK SV loop sequence of strain FA1090 was chosen because it was similar to the greatest number of Opa proteins in these two strains. The fourth loop (4L) is highly conserved among all Opa variants and a peptide that corresponds to the OpaA and OpaK 4L sequence was chosen. Antibodies were also generated against the HV<sub>2</sub> loops. Linear peptides (20 amino acids) that correspond to each HV<sub>2</sub> loop and a cyclic peptide (36 amino acids) that corresponds to the OpaB and OpaD HV<sub>2</sub> loop were synthesized and used to immunize rabbits and mice, respectively.

Ab<sub>SV cyclic</sub> and Ab<sub>SV linear</sub> recognized multiple Opa proteins by western blot and also bound the surface of Opa-positive gonococci. Ab<sub>SV cyclic</sub> recognized a greater number of Opa variants than did Ab<sub>SV linear</sub>. Ab<sub>SV linear</sub>, and Ab<sub>SV cyclic</sub> recognized gonococci that differed by up to seven amino acids from the target peptide, but neither recognized OpaC nor OpaE variants. OpaC and OpaE variants differ from the OpaA/K sequence by 11 and 12 amino acids, respectively. Results from western blot analysis of non-denatured whole cell lysates and two surface-binding assays (IFA and SBI) were

indistinguishable with respect to which Opa proteins were recognized by SV loop-specific antibodies. We conclude that the longer, cyclic peptides were better at inducing broadly reactive antibodies.

We were successful at generating broadly reactive antibodies; however, the capacity to bind Opa variants did not always correlate with the specificity of antibody function. Ab<sub>SV cyclic</sub> agglutinated OpaA but not OpaK-expressing variants, which is surprising because OpaA and OpaK have the same SV loop sequence and the OpaAK sequence was used to generate the SV loop-specific antibodies. Both OpaA and OpaK variants were recognized by Ab<sub>SV cyclic</sub> by IFA and SBI. These results suggest that, while the SV-loop sequence of OpaA and OpaK is identical, the secondary structure of the mature Opa proteins varies. OpaA and OpaK have different HV<sub>1</sub> and HV<sub>2</sub> loop sequences and the changes in structure could be due to interactions of the SV loop with either the HV<sub>1</sub> and/or HV<sub>2</sub> loop. These interactions could then alter the SV loop. None of our linear peptide-derived antibodies were able to agglutinate homologous Opa variants, a result that suggests that conformational antigens are essential to produce antibodies with agglutinating activity. Alternately, the longer cyclic peptides expressed more epitopes, and one or several of those epitopes resulted in the agglutinating capacity.

We also assessed loop-specific antibodies for bactericidal activity. Ab<sub>SV linear</sub>, Ab<sub>SV cyclic</sub>, and Ab<sub>SHV2 cyclic</sub> were not bactericidal, yet Ab<sub>HV2BD linear</sub> and Ab<sub>HV2I linear</sub> were bactericidal. We were surprised that cyclic peptide-derived antibodies were not bactericidal because studies with antibodies designed against cyclic porin-based peptides had increased bactericidal activity compared with linear peptide-derived antibodies (32). *N. gonorrhoeae* porin-based cyclic peptides induce bactericidal antibodies in our

laboratory (Garvin and Jerse, unpublished data), and therefore, we conclude that the increase in bactericidal antibodies with porin-based cyclic peptides may be antigen-specific as we did not see the same effect with cyclic peptides that correspond to Opa proteins. Alternatively, the lack of bactericidal activity could be due to a low concentration of Opa-specific antibodies in the whole mouse sera or differences in the immune response between mouse and rabbit. The bactericidal antibodies against linear HV<sub>2</sub> loop-specific peptides are affinity-purified rabbit polyclonal antibodies, in contrast to the polyclonal mouse sera that were generated with the cyclic peptides. It is unlikely, however, that the differences in host explain the lack of bactericidal activity because we detected high levels of serum IgG2a in immunized mice, which is known to activate complement. Further studies to generate high-titer antibodies against cyclic Opa-loop peptides may result in bactericidal antibodies.

We originally hypothesized that we did not see bactericidal activity with Ab<sub>SV</sub><sup>cyclic</sup> and Ab<sub>SV</sub><sup>linear</sup> because strain FA1090 is highly serum-resistant, and thus low levels of bactericidal activity were masked. Strain FA1090 is resistant to complement-mediated killing because it binds human C4BP to its porin. We therefore tested the bactericidal activity of loop-specific antibodies against a serum sensitive hybrid strain, strain FA1090<sub>F62por5-8</sub>, which expresses porin loops from strain F62 and is unable to bind C4BP. Ab<sub>SV</sub><sup>cyclic</sup> and Ab<sub>SV</sub><sup>linear</sup> were not bactericidal against strain FA1090<sub>F62por5-8</sub>. We conclude that the immunization regimens used here were not able to induce bactericidal antibodies against the conserved Opa loops even in a strain that lacks porin-mediated serum resistance.

Antibodies against the conserved loops were not bactericidal; however Ab<sub>HV2BD</sub> linear and Ab<sub>HV2I</sub> linear were bactericidal against strain FA1090. When performing bactericidal assays, the complement source needs to be considered carefully. NHS is variable and often contains naturally acquired antibodies that are cross reactive and interfere with the assay. As such, some researchers use BBS so that these factors can be controlled (156). Due to host-restrictions in complement regulation, SR strains are more sensitive to the bactericidal activity of BBS compared to NHS (132). Here, we investigated the effect of this host restriction on the bactericidal activity of Opa loop-specific antibodies and demonstrated an 8-fold increase in bactericidal activity when a non-human source of complement was used, which is consistent with the host-restriction for C4BP.

While the antibodies generated for these studies confirmed the surface-exposure of the SV loop, they did not support the 4L as being surface-accessible. Seven of eight Opa proteins were recognized by Ab<sub>4L</sub> linear by western blot, but neither surface binding assay detected Ab<sub>4L</sub> linear bound to gonococci that expressed the different Opa variants. A previous analysis of the Opa loops demonstrated that the 4L was not involved in interactions with host cells, but was involved in stabilizing the intact protein in the outer membrane (21). Thus, the 4L may not be exposed on the surface of the bacteria. Based on the studies presented here, we are unable to differentiate whether the 4L is not surface-exposed or whether our linear peptides did not produce antibodies able to recognize the conformational antigens present on intact gonococci. Further studies are warranted to generate antibodies against cyclic peptides that correspond to the 4L. Antibodies against a 4L cyclic peptide should better mimic conformational epitopes present in this loop and

may answer the question of whether the 4L is surface exposed. These studies are warranted as they would contribute to our understanding of the antigenic profile of the gonococcal outer membrane, and if the 4L is surface accessible then it provides a highly conserved target on the gonococcal surface for vaccine development.

### *Passive protection experiments with Opa loop-specific antibodies*

Development of a gonococcal vaccine has been hindered due to the lack of an animal model and no clear correlates of protection. Most gonococcal vaccines are based on the assumption that meningococcal correlates of protection will also be predictive of protection for gonorrhea. Historically, the capacity to elicit bactericidal antibodies was the gold-standard for *N. meningitidis* vaccines; however, recent studies have begun to suggest that while bactericidal activity is sufficient for protection against invasive meningococcal disease, it is not necessary. In patients who lack the terminal complement components, or vaccinated individuals that do not have bactericidal antibodies, opsonophagocytosis is protective against invasive meningococcal disease (65). In our studies, the only antibody that showed a hint of protection, was Ab<sub>HV2BD linear</sub>, which interestingly, was the only antibody tested that was bactericidal *in vitro*. These results suggest that bactericidal activity may be correlated with protection against colonization with *N. gonorrhoeae*, although further studies are still needed to confirm this observation. Continued use of the female mouse model of lower genital tract infection should allow investigations into which components of the immune response are protective against gonorrhea.

While we were unable to show protection with passively applied antibodies, studies with other STIs suggest that higher concentrations of antibodies may be required to achieve protection against infection. Vaginally applied antibodies successfully protected against herpes simplex virus type 2 (HSV-2) and SIV (136, 184, 193). Other studies used systemic antibody administration to induce persistent vaginal antibodies that were protective against *C. muridarum*, *T. vaginalis*, and HSV-2 (24, 79, 134). Studies with HSV-2 demonstrate that as little as 1  $\mu$ g of a monoclonal antibody was protective when administered vaginally immediately prior to virus inoculation (193), however 60  $\mu$ g was required when the antibody was preincubated with the virus for 15 min prior to inoculation (136). Other studies with non-human primates demonstrated that 5 mg of antibody was required to protect 75% of treated animals from acquisition of simian-human immunodeficiency virus (184). We administered 5  $\mu$ g of affinity-purified polyclonal antibodies that were preincubated for 15 min with bacteria and, based on studies with HSV-2 and HIV, we may need additional antibodies to see a protective effect.

In addition to possibly requiring more concentrated antibodies, our delivery system could be optimized to obtain sustained levels of vaginal antibodies. Our laboratory recently showed that vaginal antibodies are detectable in mice 72 hrs after intraperitoneal administration of high titer porin-loop specific antibodies. These antibodies remain detectable in the vagina for at least 96 hrs (Garvin and Jerse, unpublished data). We attempted to deliver Opa loop-specific antibodies intravenously, but were unable to detect vaginal antibodies, perhaps due to the time points chosen or the concentration of the antibodies used. As an alternative to systemic antibody delivery,

work in the microbicide field has led to the development of novel vaginal delivery mechanisms, such as vaginal rings or disks, that allow sustained levels of vaginal antibodies (107, 155). The use of a technique to allow sustained antibody levels should allow us to determine whether bactericidal antibodies are associated with protection as well as investigate the role of opsonophagocytosis in mediating protection since increased PMNs are not present in the vaginal tissue from infected mice until days 2-5 post-inoculation (172).

In summary, we demonstrated that broadly reactive antibodies can be developed against conserved Opa loops and that SV and HV<sub>2</sub> loop-specific antibodies are able to recognize multiple Opa variants. We were unable to confirm the surface exposure of the 4L as antibodies against this loop did not bind the surface of gonococci. Cyclic peptides that correspond to surface-exposed Opa loops induced agglutinating but not bactericidal antibodies as compared to linear peptide-derived antibodies. The limited duration of recovery of antibodies from the murine vagina indicates the need for better vaginal delivery systems, as most antibodies were shed within the first few hours post-administration. While no consistent protection was observed when Opa-specific antibodies were applied vaginally, these studies have laid the foundation for further studies with systemically administered, high titer antibodies. These studies are currently ongoing in our laboratory and have shown promising preliminary results (Garvin and Jerse, unpublished results).

*Opa proteins provide the gonococcus with an advantage in the  
lower genital tract of female mice*

Prior to this work, it was hypothesized that Opa proteins likely confer an advantage to the gonococcus during infection based on the demonstration that Opa-positive variants are selected during lower genital tract infection of female mice (165) and predominate in experimentally and naturally infected men (90, 92, 157, 176). There is also evidence that Opa expression is advantageous during certain stages of the menstrual cycle because in naturally infected women the Opa phenotype of cervical isolates varies with the menstrual cycle (90). In female mice, the recovery of Opa variants is cyclical with three distinct phases observed (165). Here we show that Opa proteins do provide an advantage during infection, and that reproductive hormones are involved in the cyclical recovery pattern observed in females.

The original description of the cyclical recovery pattern by our lab was based on mice treated with a 5 mg slow-release  $17\beta$ -estradiol pellet (165). To ensure that the observed recovery pattern was not due to the administration of high levels of exogenous estradiol, we tested mice that were treated with 1.5 mg water soluble  $17\beta$ -estradiol or left untreated. Regardless of the treatment regimen, mice showed initial selection for Opa protein expression following inoculation with mostly Opa-negative variants, followed by a decline in Opa-positive variants in the mid-phase and then an increase in the recovery of Opa-positive variants in the late phase. *In vitro* growth kinetics in broth that contains concentrations of  $17\beta$ -estradiol that correspond to the serum estradiol levels in the different models did not result in selection for Opa-positive variants or a change in



growth rate. These results suggest estradiol alone is not directly responsible for the cyclical Opa recovery pattern we have observed, and that another factor(s) present in the female lower genital tract provides an advantage to Opa-positive gonococci during infection.

Clinicians and researchers have noted for years that there appears to be a link between different aspects of gonorrhea and the female reproductive cycle (5, 74, 85, 90, 96, 104, 121). To further confirm the potential link between the cyclical Opa recovery pattern and the reproductive cycle, we infected 17 $\beta$ -estradiol-treated ovariectomized (Ov<sup>-</sup>) or intact mice with predominantly Opa-negative variants of strain FA1090. Both Ov<sup>-</sup> and intact mice showed selection for Opa-positive variants in the early phase. Interestingly, Ov<sup>-</sup> mice did not show the cyclical Opa recovery pattern but maintained selection for Opa-positive variants throughout the experiment. These results suggest that the factor(s) responsible for the decline of gonococci during the mid phase is regulated by the ovaries.

Consistent with the link described in Chapter 3 between recovery of gonococci and the female reproductive cycle, it was shown in the 1940s that naturally infected women cultured throughout one complete menstrual cycle demonstrated reduced recovery of gonococci from days 22-25 of the cycle (104). Other studies showed increased recovery of gonococci within the first seven days of the menstrual cycle (96, 121, 178) and variations in the recovery of opaque and transparent colonies throughout the menstrual cycle (90). As further studies are undertaken, it is becoming clear that the reproductive cycle may also affect susceptibility to viral infection or disease progression (25) and that gonococcal pathogenesis differs between men and women (51). A greater

appreciation for gender differences during infection and disease progression is beginning to evolve. Continued progress in this area will require adaptation of conventional dogma and the development of new model systems with which to study the female host.

The use of defined Opa mutants that express no Opa proteins, FA1090*opaA-K*, or constitutively express OpaB, FA1090*opaA-K(B+)*, allowed detailed analysis of the three phases of Opa recovery that are observed during wild type infections. Based on the strong selection for Opa proteins in the early phase, we hypothesized that an Opa-deficient strain might be unable to establish infection in female mice. Surprisingly, the Opa-deficient strain was able to colonize the murine genital tract, although at reduced levels compared to the OpaB-expressing strain on day 1. In the mid-phase (days 4-7) during which Opa-negative variants predominate in wild type infections, both the Opa-expressing and Opa-deficient strain showed declines in the number of CFU recovered; these results suggest that the mid-phase may be Opa-independent. During wild type infection, the mid-phase is composed of predominantly Opa-negative variants, however non-competitive infections, such as that used to compare the recombinant strains, may not be sensitive enough to demonstrate an advantage for the Opa-deficient strain. The decline of CFU in the mid-phase suggests bacterial clearance by the host, or possibly tissue invasion. Identification of the factor involved in the decline of recoverable gonococci in the mid phase may prove to have therapeutic potential as both Opa-positive and Opa-negative gonococci appeared to be susceptible to this factor.

Perhaps the most important conclusion made from studies using the defined Opa mutants is that Opa expression, while not essential, provided the gonococcus with a long-term fitness advantage. Mice infected with an Opa-expressing strain remained colonized

longer with 69% of mice still culture-positive on day 14, compared to only 38% on day 14 for mice infected with the Opa-deficient strain. The increased persistence of an Opa-positive strain could greatly influence the ability of gonococci to transmit from one infected individual to another.

The expression of multiple Opa proteins has been hypothesized to be a mechanism of immune evasion, whereby changes in the outer membrane profile prevents a robust anti-gonococcal immune response. At late time points during gonococcal infection of mice and men, gonococci that express multiple Opa proteins are observed (92, 165). If expression of multiple Opa proteins is required for sustained colonization in the late phase, strain FA1090*opaA-K* (*B+*), which can only express a single Opa protein, would potentially be less fit than wild type gonococci during a 14 day experiment. However, based on our results with strain FA1090*opaA-K* (*B+*), it does not appear that the capacity to express multiple Opa proteins is required for long-term colonization of female mice. To date, it is unknown what advantage is gained by the expression of multiple Opa proteins, and several factors may be involved in selecting for this phenotype.

#### *Investigation of selective factors for Opa-positive gonococci*

The best characterized function for Opa proteins is the ability to mediate adherence to and invasion of human tissue culture cells. Studies with tissue culture cell lines have identified several members of the human CEACAM family and HSPG molecules as receptors for Opa proteins (reviewed in (131)), although not all Opa

proteins interact with these receptors (129). The tissue distribution of CEACAMs does not correlate with the predominant sites of gonococcal infection and is further evidence that CEACAMs may not be involved in gonococcal pathogenesis. Analysis of primary male urethral epithelial cells or normal cervical and fallopian tube cells demonstrate limited or no CEACAM expression (75, 177).

While mice express CEACAM1, comparison of the human and murine CEACAM1 structures suggest that murine CEACAM1 will not support Opa binding due to changes in several critical amino acids (165, 185, 186). In support of these observations, no increased adherence to cultured murine cells was observed for an Opa-expressing strain, as compared to an Opa-deficient strain. We hypothesized that if Opa proteins were providing a colonization advantage in mice, then in the absence of human CEACAMs, an HSPG-binding Opa protein would have a competitive advantage over a CEACAM-binding Opa variant. To test this hypothesis, we infected mice with a predominantly Opa-negative inoculum spiked with equal numbers of OpaI (HSPG and CEACAM-binding) or OpaB (CEACAM-binding) variants and monitored the Opa phenotype recovered during the early phase. The OpaI gonococci were not preferentially selected for over the OpaB gonococci (Cole and Jerse, unpublished data) even though HSPG are expressed in the murine genital tract (77). We conclude that the advantage seen for Opa-positive gonococci in the early phase is unlikely to be due to increased colonization and that HSPG-mediated adherence is not detectable in mice.

In addition to CEACAM and HSPG-mediated invasion, Edwards recently reported that interactions between porin, pilus, and host iC3b with the CR3 receptor resulted in increased uptake into primary human cervical epithelial cells (52, 53). While

this invasion does not involve Opa proteins, the link to complement was intriguing in light of the clear association between some Opa proteins and complement resistance. While we did not directly investigate whether CR3-dependent invasion occurs in mice, the low level of invasion into murine cell lines does not support a role for CR3-dependent invasion during murine infection. CR3-dependent invasion may be therefore be host-restricted.

The cyclical recovery pattern could be due to invasion of gonococci into the genital tissue. The finding that upper reproductive tract (49) and DGI isolates are often transparent (Opa-negative) (133) suggests that Opa-negative gonococci may be more invasive. If Opa-negative bacteria invade better in the early and late phases, then we would preferentially recover Opa-positive gonococci by vaginal swab in these phases, which is what we have observed previously and in these studies (Figs. 4 and 13). We did not see invasion in monolayers of murine tissue culture cells, but perhaps the receptor is only expressed on polarized cells. Attempts to polarize murine cells were unsuccessful and we were unable to investigate CEACAM-independent invasion across a polarized human epithelial layer due to technical limitations with the HEC1B cell line. Further studies are needed to investigate the role of CEACAM-independent invasion in the murine genital tract, as we hypothesize that CEACAM and HSPG are not contributing to bacterial invasion. Immunohistochemical staining of vaginal tissue from wild type-infected mice could be used to determine whether Opa-positive gonococci invade the tissue in the mid phase.

Our tissue culture studies with murine-derived cell lines do not support increased adherence or invasion by Opa-positive gonococci through murine CEACAMs, HSPG, or

a previously undefined receptor, and we hypothesize that the function of Opa proteins is to mediate evasion of innate factors. *In vitro* studies performed here with strain FA1090 confirm that Opa proteins increase resistance to the bactericidal effects of NHS when the inherent SR of this strain is genetically removed. As porin-mediated SR does not occur in mice this seemed like a relevant avenue of investigation. We observed a hierarchy of complement resistance with OpaB and OpaEK conferring consistent, but not statistically significant, increases in bactericidal<sub>50</sub> titers as compared to Opa-negative variants. OpaF variants and variants that express OpaF and OpaA or OpaF, OpaA and OpaEK demonstrated the highest level of complement resistance and were greatly increased over Opa-negative variants. While *in vivo* studies have eliminated complement as the sole factor responsible for early phase selection, perhaps in the late phase the expression of multiple Opa proteins provides resistance to complement. During the late phase we often recover colonies that express multiple Opa proteins simultaneously, and we commonly see OpaF and OpaEK as part of these multiples. Studies to compare the recovery of wild type gonococci, which are able to express multiple Opa proteins simultaneously, in the late phase of complement-deficient mice would be interesting to address the contribution of multiple Opa-expressing gonococci to pathogenesis.

We determined that the selective factor for Opa proteins is directly or indirectly under ovarian control, but additional studies in untreated mice may provide insight to what stage of the estrous cycle regulates this factor. Shown in Figure 19A are stained vaginal cells from uninfected mice in each of the four stages of estrous. Untreated mice are susceptible to gonococcal infection while in proestrus and remain colonized until they cycle out of estrus (42). Here, we infected untreated mice while in the proestrus stage of

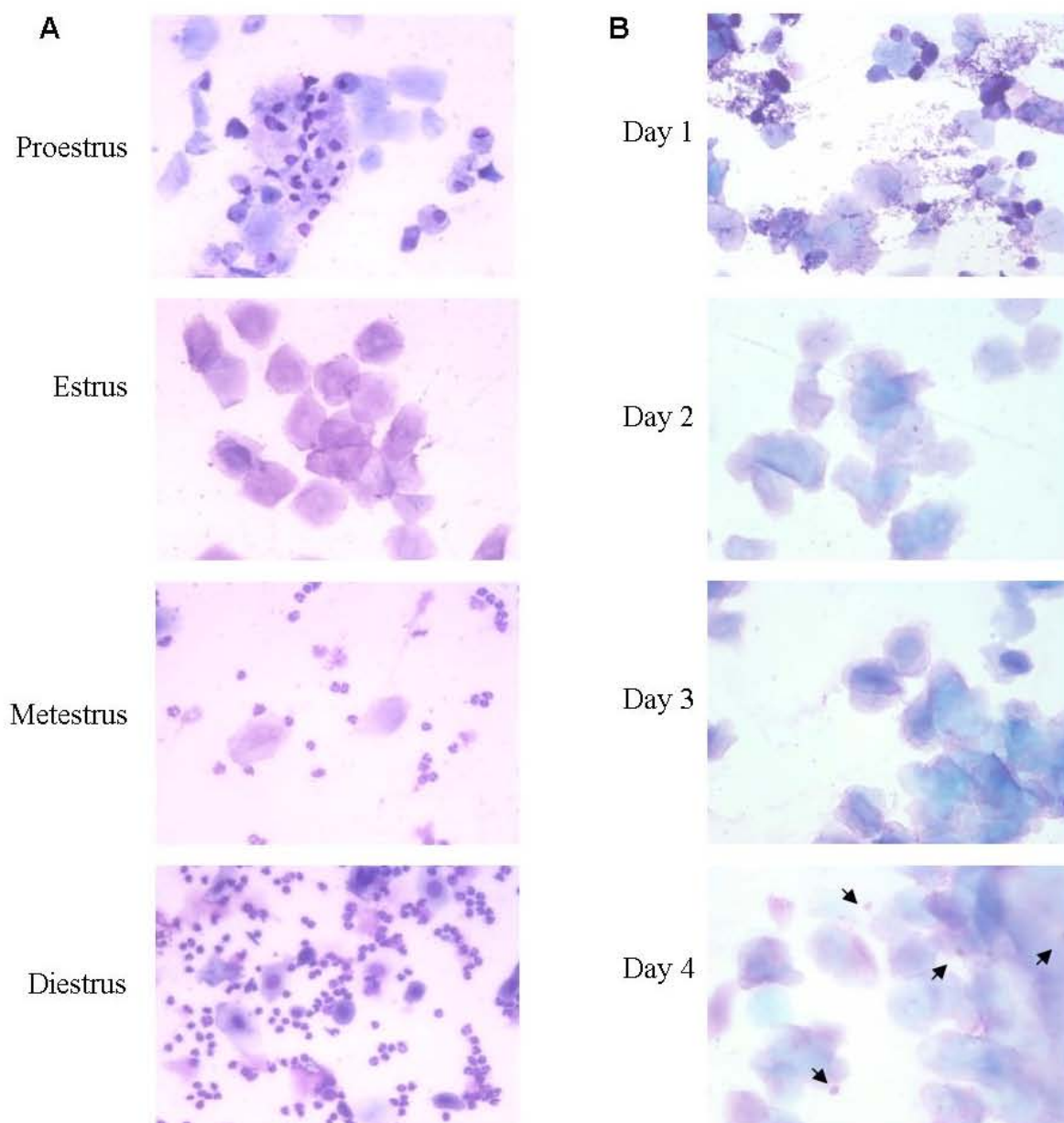
the estrous cycle, in which the vaginal mucosa is composed of nucleated epithelial cells and very few squamous epithelial cells. We observed cyclical recovery of Opa variants in untreated mice and shown in Figure 19B are vaginal smears from days 1-4 of infection from a mouse that exhibited the cyclical Opa recovery pattern. On day 1 the mucosa is characteristic of late proestrus/early estrus, on days 2 and 3 this mouse is in estrus, and on day 4 is in late estrus/early metestrus, as evidenced by the start of a PMN influx. On days 2 and 3 while this mouse was clearly in estrus, less than 17% of the gonococci recovered were Opa-positive. On days 1 and 4, 42% and 83% of the CFU were Opa-positive, respectively (see Fig. 13B). These results suggest that the selective factor(s) may be expressed in late proestrus/early estrus and late estrus/early metestrus, but not during the middle of the estrus stage. It would be interesting to culture mice on the day of inoculation (day 0) and if this hypothesis is correct I predict that greater numbers of Opa-positive gonococci would be observed late on day 0, compared to day 1 where mice are transitioning from proestrus to estrus. Analysis of vaginal gene expression in mice by DNA microarray or proteomics on day 2 and 3, compared to day 4 may help identify the selective factor.

We have eliminated several factors as the cause for the cyclical recovery pattern and hypothesize that multiple factors may be involved in creating an environment that is favorable to Opa-positive variants. I propose that the selection for Opa-positive variants is due to hormonally regulated innate factors that create an environment that is favorable to Opa-positive gonococci. Beta-defensins are increasingly being recognized as important innate factors and are expressed in humans and mice (161). Human beta-defensins (HBD) have a distinct and discrete menstrual cycle-dependent expression

**Figure 19. Stained vaginal smears from normal cycling mice and a mouse infected with *N. gonorrhoeae*.** (A) Vaginal smears obtained from normal cycling mice in each of the four stages of the estrous cycle. During proestrus occurs the vaginal mucosa is thin and is characterized by predominately nucleated epithelial cells with very few squamous cells. As estrogen levels increase in estrus, cell proliferation occurs and squamous epithelial cells predominate. Following estrus, PMNs influx in metestrus and increase dramatically in diestrus where they are the predominant cell type. (B) Stained vaginal smears on days 1-4 from a mouse that was not treated with estradiol and was inoculated with *N. gonorrhoeae* when in the proestrus stage (day 0). The pie charts in Fig. 13B demonstrate the Opa phenotype in this mouse on days 1-4. Arrows on day 4 indicate PMNs, which are indicative of the beginning of metestrus.



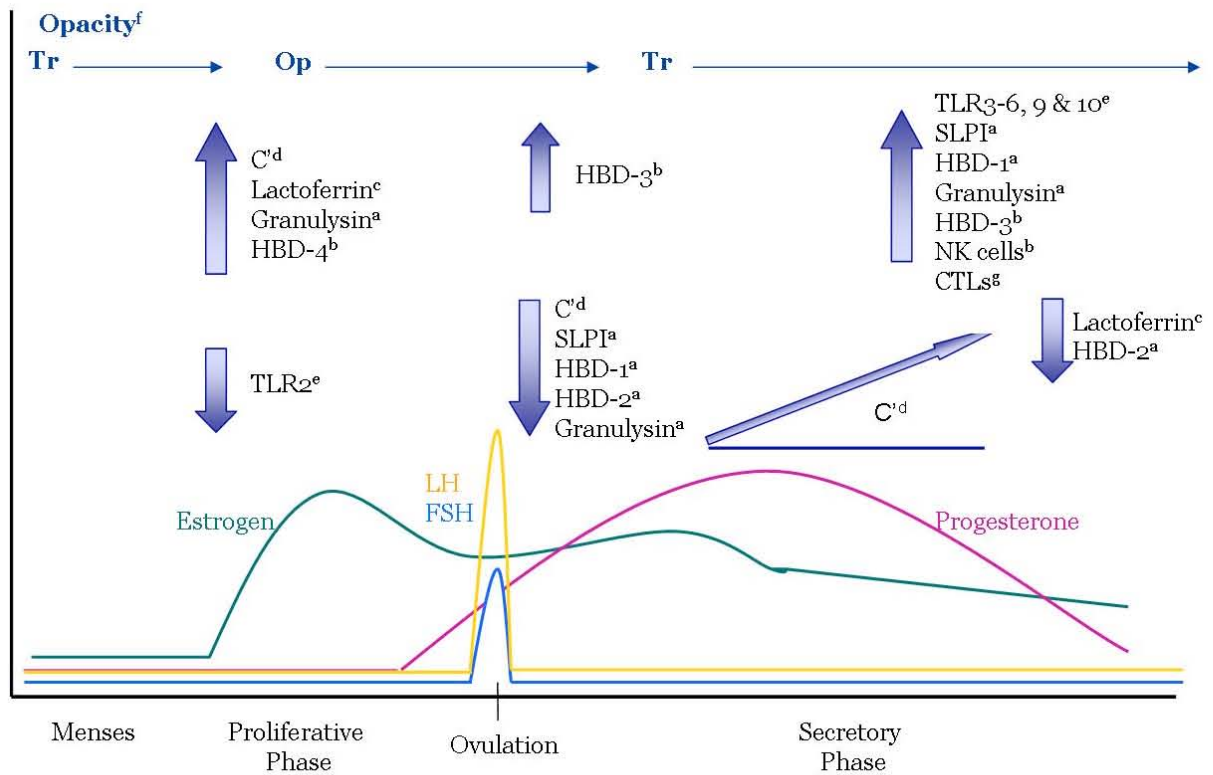
Figure 19. Stained vaginal smears from normal cycling mice and a mouse infected with *N. gonorrhoeae*.



profile in the endometrium (102). These antimicrobial peptides may contribute to the cyclical recovery pattern. Singh *et al.* recently demonstrated a synergistic bactericidal effect against *E. coli* with lactoferrin, SLPI, and lysozyme (167), all of which are present in the genital tract in a menstrual cycle-dependent fashion (72, 102). Some of the factors that are hormonally regulated and could potentially be involved in the cyclical recovery pattern are shown in Figure 20. While studies here eliminated complement as the sole selective factor in the early phase, it may be involved in the late phase or contribute to the early phase in conjunction with other factors.

**Figure 20. Factors that are differentially expressed during the female reproductive cycle may contribute to the cyclical recovery pattern of Opa variants.** Many factors fluctuate during the female reproductive cycle and some of those factors that may be involved in cyclical Opa recovery are indicated here. Arrows indicate up or down regulation of factors during the proliferative and the early and late secretory phases of the menstrual cycle. Abbreviations: C', complement; HBD, human beta-defensin; TLR, toll-like receptor; SLPI, secretory leukoprotease inhibitor; CTL, cytolytic T cell.

Figure 20. Factors that are differentially expressed during the female reproductive cycle may contribute to the cyclical recovery pattern of Opa variants.



a. (55); b. (102); c. (33); d. (76, 112); e. (3); f. (90); g. (72).

### *Future Directions*

The studies presented here leave several questions to be answered: i.) What factor gives Opa-positive gonococci an advantage in the early and late phases? ii.) Is the mid-phase truly Opa-independent and what factor causes a decline in gonococci in the mid-phase? iii.) What is the contribution of multiple Opa-expressing gonococci in the late phase? iv.) What role, if any, does Opa-mediated complement resistance play *in vivo*? Experiments that would address the latter two questions were presented earlier in this chapter.

To identify the selective factor for Opa-positive gonococci and investigate the factors illustrated in Figure 20, several additional studies need to be undertaken. An analysis of changes in host gene expression during the three phases of the cyclical Opa recovery pattern should identify a subset of factors that may contribute to the recovery pattern. Gene expression patterns in estradiol-treated or untreated mice should reveal the same set of factors. Another approach is to use flow cytometry to analyze whether proteins present in vaginal washes from the early, mid and late stages of the cyclical recovery pattern preferentially bind to Opa-positive or Opa-negative gonococci. Flow cytometry is limited by the need for antibodies to factors present in vaginal washes and is not an unbiased screen but is dependent on the choice of antibodies and, as such, some factors might be overlooked. It is important to remember while interpreting results from these methods that multiple factors may be involved, only some of which may be identified by each method. After identification of potential factors, *in vitro* analyses for

preferential antimicrobial activity against Opa-positive and Opa-negative bacteria should be performed. Combinatorial effects between potential factors should also be investigated as synergism may occur *in vivo*. Identification of the factors that cause the cyclical recovery pattern will advance our understanding of gonococcal pathogenesis as well as the innate immune defenses in the female reproductive tract.

In summary, the results presented here are a significant step forward in our understanding of gonococcal pathogenesis as it relates to Opa proteins and a female host. *In vitro* studies have implicated Opa proteins as being involved in increased complement resistance and adherence to and invasion of host cells, but here we have shown that these two factors are not likely to cause the cyclical recovery pattern observed in mice. Our findings clearly demonstrate that the ovaries dramatically affect the pathogenesis of *N. gonorrhoeae* and highlight the importance of studying the female host. These studies are also the first to demonstrate an *in vivo* advantage for Opa expression in that although an Opa-deficient strain can colonize the genital tract, Opa positive variants persist longer and thus may provide increased opportunities for transmission to a new host. There appears to be a novel function of Opa proteins in the female reproductive tract that remains to be elucidated.

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